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THE EFFECTS OF INTERFERON AND  
DOUBLE-STRANDED RNA

BY

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I declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree, and that the work is my own except where specifically acknowledged.

*Jonathan A. Cooper*

SUMMARY

1. The effects of dsRNA on interferon-treated and control cells, and on various cell-free systems, have been characterised, and discussed in relation to theories on the role of dsRNA in the control of cell functions.
2. The double-stranded homopolymer, poly rI.rC, used for most of these experiments, was characterised in terms of its resistance to ribonucleases and its behaviour on cellulose column chromatography.
3. The effects of various homopolymers on protein synthesis in the reticulocyte lysate were studied and related to their published effects on intact cells. The apparent requirement for high molecular weight is discussed in relation to measurements of structural stability.
4. The cytotoxic effect of dsRNA on interferon-treated L929 cells was characterised, and a proportion of the cells resistant to the toxicity was found to exist. No evidence for a specific biochemical trigger for toxicity could be found.
5. A cell-free protein synthesising system from monolayer grown L929 cells was developed and characterised.
6. An assay for an interferon and dsRNA dependent<sup>e</sup> inhibitor of protein synthesis was developed using reticulocyte lysates. Interferon-treated cell cytoplasm was capable of forming an inhibitor of protein synthesis when incubated with dsRNA and ATP, which inhibited a specific step in protein synthesis initiation. The relevance of this inhibitor to changes in protein phosphorylation is discussed.
7. There was no evidence that the inhibitor is generated when interferon-treated cells are exposed to dsRNA, so the inhibitor may not be involved in the cytotoxic effect.

ABBREVIATIONS

ATP	adenosine 5' triphosphate
BEV	bovine enterovirus
BSA	bovine serum albumin
°C	degrees Centigrade
Ci	Curie ( $2.2 \times 10^{12}$ decompositions/min)
cpm	counts per minute
CS	calf serum
CTP	cytidine 5' triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N,N'-tetraacetic acid
EMC	encephalomyocarditis
FBS	foetal bovine serum
F met tRNA <sub>F</sub>	formyl methionyl tRNA (initiator)
g	gramme
g	gravity (centrifugal force at average radius)
GMEM	minimal eagles medium (Glasgow modification)(section 2.2.1).
GTP	guanosine 5' triphosphate
HAU	hemagglutination unit
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HnRNA	heterogeneous nuclear RNA
hr	hour
IF-E2	initiation factor-E2
INAS <sub>50</sub>	50% inhibition of nucleic acid synthesis
l	litre



log	logarithm (to base 10)
m	metre
M	molar
met tRNA <sub>F</sub>	methionyl tRNA (initiator)
min	minute
mRNA	messenger RNA
ND	not done
NDS	naphthalenedisulphonic acid (sodium salt)
NDV	Newcastle Disease virus
NURB	Neville upper reservoir buffer (section 2.6.2)
OD <sub>x</sub>	optical density at x nm
PBS	phosphate buffered saline (section 2.2.1)
pH	negative log of hydrogen ion concentration (M)
poly A(+) RNA	poly rA containing RNA
poly rC	polyribocytidylic acid
poly rI	polyribinosinic acid
poly rI.rC	double-stranded complex of poly rI with poly rC
POPOP	1,4-di (2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid
(±)RNA	viral RNA of message (+) or complementary (-) sense
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit
S-10	supernatant from 10000 g spin
SDS	sodium dodecylsulphate

SFV	Semliki Forest Virus
ss	single-stranded
SV40	Simian Virus 40
TCA	trichloroacetic acid
TNE	see section 2.4.1.
TLES	see section 2.4.1.
tris	tris-hydroxymethyl amino methane
tRNA	transfer RNA
ts	triple-stranded
u	activity unit; for interferon, international unit
vRNA	viral RNA
VSV	Vesicular Stomatitis Virus
v/v	volume for volume
w/v	weight for volume

## SECTION 1

### INTRODUCTION

# 1.1. BACKGROUND

The subject of this research (section 1.7) was to relate some of the in vivo and in vitro effects of interferon and dsRNA in the hope of elucidating the cause of the cytotoxicity of dsRNA for interferon-treated cells. Before embarking on a detailed review of the relevant literature, and in order to put the project in context, this section will briefly outline the current state of interferon research. A minimum of references will be given, but some of the more contentious points will be discussed in detail elsewhere.

Interferon<sup>was</sup> ~~were~~ first recognised by Isaacs and Lindenmann (1957) as being a substance which was released by cells which had been treated with virus, and which was able to prevent virus replication in other cells. The early evidence (see Cantell 1973) showed that interferons were a group of glycoproteins induced in cell culture or animals by viruses, polynucleotides, and some other compounds, able to protect homologous cells against infection by a range of viruses by blocking a stage of virus replication subsequent to penetration and uncoating. Attempts at purification met with little success, and it was clear that biological activity resided in only a small fraction of the "interferon" preparation. The literature was confused with artefacts resulting from the use of crude preparations and from the complex results obtained in experiments using whole animals. The use of purer preparations of interferon, and of tissue culture cell systems has facilitated work on the molecular aspects of interferon, with the following tentative conclusions:

- a) Interferons are induced in cell culture by a range of active and inactive viruses, and by polyribonucleotides having certain molecular characteristics (de Clercq 1974). Other inducers probably act on cells of the immune system.
- b) Production of interferon requires the induction of a cellular gene and the transcription and translation of interferon mRNA. The mRNA can be translated in heterologous systems and the gene, or genes, localised to certain chromosomes. The production is

regulated either at transcription or translation by unknown processes, and "superinduction" can occur by judicious use of metabolic inhibitors (Reynolds et al 1975, Creaghan et al 1975, Tan et al 1970).

- c) Interferons from one source can be very heterogeneous, and different cell types from the same species of animal may produce different spectra of molecules. The antiviral activity of a preparation is always expressed in homologous cells, but may also protect cells from unrelated animals. (Stewart and Desmyter 1975).
- d) Interferons have very high specific activities and require special methods for purification. It is possible that some interferons have now been purified to homogeneity. Estimates suggest that the antiviral activity of a pure interferon could approach  $10^9$  u/mg protein. This high specific activity implies that only a few molecules are required to protect one cell (Knight 1975).
- e) The development of an antiviral state requires interaction of interferon with the cell followed by various metabolic processes (de Clercq and Stewart 1973).
- f) Replication of a range of viruses is inhibited in interferon-treated cells to a greater or lesser extent. There may be several lesions in a given virus/cell system, or all the effects may stem from one primary lesion.
- g) Extensively purified preparations of interferons have other, non-antiviral activities on recipient cells. These include inhibition of cell growth, priming of interferon induction, and enhancement of the toxicity of dsRNA (de Clercq and Stewart 1973).

The remainder of this introduction will survey the literature on the biological activities of interferon and dsRNA, taking into account work published up to mid 1976.

## 1.2. INTERACTION OF dsRNA WITH CELLS AND INTERFERON INDUCTION

This section will consider the interaction of dsRNA with the cell surface, and relate this to its effects on intact cells, in particular interferon induction. Besides inducing interferon, dsRNA also has ~~and direct~~ antiviral effect on the recipient cell, and this has been shown to depend on the production of extracellular interferon (Schafer and Lockart 1970, Vengris et al 1975), so the two effects can be considered together.

### Structural requirements for the inducer

dsRNA was first shown to be an interferon inducer in 1967 when the active component in filtrates of Penicillium funiculosum was found to be dsRNA (Lampson et al 1967) and reovirus and synthetic dsRNAs were also shown to be active (Tytell et al 1967; Field et al 1967). Since then a whole range of chemically modified polymers have been studied, and certain molecular requirements have become apparent (de Clercq 1974):

- a) Stable secondary structure. Most duplexes with a melting temperature of 60°C or above (0.15M salt) are good inducers, providing other criteria are met. However, complexes of poly C<sup>7</sup>A with poly rT and poly br<sup>5</sup>U are exceptions (de Clercq et al 1974), perhaps because the helix form is distorted (A.M. Bobst, personal communication 1976).
- b) High molecular weight. If the length of one, or both, strands of a helix is reduced, the inducing ability is also reduced. The critical range for this is from 2S to 5S, and when both strands are greater than 5S, interferon induction is maximal (Tytell et al 1970; Niblack and McCreary 1971).
- c) Resistance to ribonucleases. As the assay for interferon induction requires the inducer to reach the cell, it is clear that the dsRNA must resist attack by nucleases present in the tissue culture fluid and on the cell surface. The increased induction by dsRNA complexed with polycations such as DEAE dextran and poly-L-lysine (Dianzani et al 1968) may result from protection of the dsRNA against nuclease degradation (Pitha and Carter 1971). Annealing dsRNA in media

containing divalent metal cations may increase induction by melting out local irregularities in structure, hence increasing nuclease resistance (de Clercq and Merigan 1971).

- d) Presence of 2' hydroxyl groups. Substitution of 2' (ribose) hydroxyl groups on either strand by a range of groups (H, F, Cl, N<sub>3</sub>, OCH<sub>3</sub>, OCOCH<sub>3</sub>, etc.) produces inactive molecules. DNA.RNA hybrids and dsDNA are inactive. Kleinschmidt (1972) has speculated that this is due to a change in flexibility of the helix.

These four criteria overlap considerably, and varying one parameter often varies others. However, it does seem that these criteria are adequate to define a molecule as a good interferon inducer (de Clercq 1974). Moreover, the same requirements probably apply for other biological activities of dsRNA: DNA synthesis in HeLa cells is inhibited by dsRNA, but not ssRNA or dsDNA (Teng et al 1973); interferon-enhanced cytotoxicity is observed with dsRNA, but not ssRNA or dsDNA (Stewart et al 1973a); antisera raised against dsRNA recognise homopolymers able to induce interferon (Johnston et al 1975); and the effects of dsRNA on the reticulocyte lysate protein synthesising system are not shown by ssRNA or dsDNA (Hunter et al 1972).

It is thus likely that high molecular weight, stability, nuclease resistance, and 2' hydroxyl groups are the minimum requirements for cellular receptors to distinguish dsRNA from normal cellular nucleic acids.

A complication arises when poly rI.rC complexes are considered. Instead of the simple size requirements outlined above, the size of the poly rI strand appears to be more important than the size of the poly rC strand (Tytell et al 1970; Carter et al 1972; Mohr et al 1972). Provided that the length of the poly rI strand (or the distance between modified bases in that strand) exceeds about 500 nucleotides, the length of the poly rC strand can be reduced to 70 nucleotides without affecting interferon production, but the converse is not true. The failure of Niblack and McCreary (1971) to observe this effect has been attributed to the polydispersity of their polynucleotides (de Clercq 1974).

This requirement is also shown for other biological activities of poly rI.rC: interferon-enhanced cytotoxicity and curing of L1210 cell leukaemias (Stewart and de Clercq 1974; Mohr et al 1972). These similarities can be explained when the thermal stability of the duplexes is considered (Mohr et al 1972), because a long poly rI strand is more important than a long poly rC strand in conferring a high melting temperature. However, Carter et al (1972) showed that even when matched for equal thermal stability, complexes of poly rI with oligo rC were more active than oligo rI. poly rC. Both these complexes were unstable under the conditions for induction and were assayed in the presence of poly-L-lysine, so the results are not strictly comparable with those obtained using poly rI.rC alone.

Some other evidence suggests that the poly rI strand is intrinsically more important than the poly rC. De Clercq and de Somer (1972) showed that although poly rI and poly rC are normally inactive, they can combine at the cell surface when added sequentially to cells, and thus induce interferon. This was most marked when poly rI was added before poly rC, rather than vice versa. In the presence of polycations, poly rI may induce even in the absence of poly rC (Pitha and Pitha 1974), and poly rI complexed to a hydrocarbon carrying cytosine residues on alternate carbon atoms (poly (1-vinyl cytosine)) is a good inducer (Pitha and Pitha 1971).

These activities of poly rI might reflect its ability to form multi-strands, for, even though unstable under the ionic conditions used for induction (Michelson et al 1967), macromolecular receptors could favour multistrand formation. The ability to form multistrands has been shown to correlate with the effects of a range of ssRNAs on the template activity of isolated nuclei or chromatin (Brown and Coffey 1972), suggesting that multistrand formation may occur in the cell.

The fact that interferon induction, interferon-enhanced cytotoxicity, and possibly protein synthesis inhibition, are all caused by molecules having the same structural features does not necessarily mean that the same



receptors are involved. It is likely that the properties recognised in the dsRNA are the minimum required to distinguish it from normal cellular polynucleotides, so the selection pressure for such specificity would be high.

#### Binding sites and uptake of dsRNA

Having considered the molecular requirements for recognition of a dsRNA by cellular receptors, it is necessary to consider the nature of the interaction of dsRNA with the cell. Again, this has been investigated primarily in the context of interferon induction.

De Clercq et al (1974) used interferon induction as a marker for binding to the induction-specific receptor. When ssRNA was used to compete for dsRNA binding sites, tsRNA was formed even though the ionic conditions would normally have favoured dsRNA (de Clercq et al 1975b). Interferon was not induced, and they concluded that tsRNA bound more strongly than dsRNA to the induction-specific receptor, so tsRNA was stabilised. This adds another molecular requirement to the list of criteria for interferon inducers - that disproportionation of dsRNA to tsRNA should be unfavourable (de Clercq et al 1974).

The  $tsRNA > dsRNA > ssRNA$  affinity is not found when binding is performed in the cold (Johnston et al 1976). Instead, ssRNA probably does bind to the specific receptor, because cells carrying labelled poly rI.rC lose some label and the capacity to produce interferon when treated with ssRNA. It is interesting that sequentially added poly rI and poly rC are apparently more resistant to displacement by poly rC (Johnston et al 1976).

Apart from these attempts to characterise the binding properties of the specific receptor, studies on the interaction of dsRNA with the cell have concentrated on two questions. What is the chemical nature of the receptor? and, does this receptor trigger induction or merely provide a transport system for dsRNA? Experimental approaches have made use of modification of the cell surface, following the fate of radioactive dsRNA, and insolubilised dsRNA.

Bausek and Merigan (1969) showed that labelled poly rI.rC bound to cells at 4°C, and this was enhanced by polycations. The main problem is that the bulk of this binding is not required for induction. Thus, induction can be prevented by concanavalin A (Harper and Pitha 1974), mild enzyme treatment (Pitha et al 1974), or salt washing (Johnston et al 1976), yet all these procedures leave a considerable proportion of dsRNA bound to the cell, implying that only a small amount of binding is specific.

When cells carrying dsRNA are warmed, a small proportion of the dsRNA becomes ribonuclease resistant (Bausek and Merigan 1969). However, it is not clear whether this fraction is responsible for induction. Schell (1971) showed that poly rI was taken up in preference to poly rC when ascites tumour cells were treated with poly rI.rC. If the relative importance of the poly rI strand for induction also applies with these cells, these results might imply that intracellular RNA triggers induction.

Good evidence that dsRNA can enter the cell without degradation is provided by the observation that mengovirus RF is infectious (Perez-Bercoff et al 1974a). However, most of the polynucleotides entering the cell are probably degraded and may be recycled into cellular RNAs. Clearly, this limits the usefulness of autoradiography for localising internal dsRNA, because intracellular label must be shown to be not only acid insoluble, but also to be dsRNA. Those cell types requiring most dsRNA for induction are also most active at degrading it (de Clercq and Stewart 1974).

By contrast with these experiments, the use of insolubilised dsRNA attempts to demonstrate that dsRNA can induce without needing to enter the cell. However, this approach has been of limited usefulness, because all supports are "leaky" and interferon induction could be explained either by local high concentrations of free dsRNA, or by the high efficiency of dsRNA presented in a form readily absorbed onto cells (de Clercq and de Somer 1974a). Indeed, Hutchinson and Merigan (1975) concluded that induction only occurred when some dsRNA was lost from the support, that is, when some free dsRNA was available.

Rather than attempting to ask whether dsRNA can induce interferon from the outside of the cell, it would perhaps be more profitable to test whether dsRNA can induce from the inside. This could be tested by microinjection with liposomes or red cell ghosts to deliver dsRNA to the inside of the cell, with ribonuclease added to the medium to destroy any dsRNA which might escape to the outside. In the long term, development of cell free transcription systems might show whether interferon mRNA synthesis is induced by dsRNA or a "second messenger".

The mechanism of interferon induction by viruses is still more uncertain and not very relevant to this discussion. However, some points concerning the possible role of intracellular dsRNA can be made:

- a) RNA viruses are generally better inducers than DNA viruses (de Clercq and Stewart 1973). Herpesviruses do not induce, and RNA extracted from infected cells was unable to induce interferon (Perez-Bercoff et al 1973).
- b) Correlating interferon induction with the presence of dsRNA in various restrictive systems does not usually give clear cut results. Two situations can arise. dsRNA may be detectable but no interferon is induced, either because the dsRNA is in a separate compartment, or because shut off of host cell protein synthesis is too rapid (eg. NDV in chick cells, Gandhi and Burke 1970; Dubois 1974), or because dsRNA is not the inducer. Alternatively, dsRNA may not be detectable even though interferon is induced. Here the problem is with limits of detection - Atkins and Lancashire (1976) found some Sindbis virus ts mutants which did not produce dsRNA were able to induce interferon in chick cells with wild-type kinetics. In this case salt precipitation, cellulose columns, radioimmunoassay (G.J. Atkins, personal communication 1975) or protein synthesis inhibition (J.A.C. and G.J.A., unpublished results) were all used to test for dsRNA, but all had a lower limit of detection of 1-5% of wild-type levels.

- c) It is difficult to think of any other intermediate in virus replication besides dsRNA, which is common to all the viruses able to induce interferon. Alternative theories thus rely on different inducers functioning in different systems, for example progeny virus (Lai and Joklik 1973) or an early event leading to cytopathic effects (Atkins 1976).

If dsRNA is the inducer in virus-infected cells, it suggests that exogenous dsRNA either induces by a different route, directly at the outer face of the membrane, or needs to be transported across the membrane to interact with an intracellular receptor. At present, the only way of excluding one of these possibilities would be to find a situation where less than one molecule of dsRNA enters each cell yet interferon is still induced. Because of the large amount of non-specific binding, it is difficult to see how induction at the cell surface could be excluded. Purification and characterisation of the surface receptor/transport system would be useful, but before this is practical some means of chemically distinguishing the "specific" and "non-specific" sites must be found - there is no reason to suppose that the "specific" sites will be of higher affinity than the others, and the "non-specific" sites may even be specific for other activities of dsRNA!

### 1.3. INTERACTION OF INTERFERON WITH CELLS AND THE ANTIVIRAL STATE

This section will consider the interaction of interferon with the cell surface, the subsequent changes in the cell, and the stage in virus replication which is blocked. Other sections will deal with the changes observed in vitro and with the non-antiviral effects of interferon.

The action of interferon on a cell can be split into two stages - the initial binding and interaction with the cell surface, and the induction and development of the antiviral state. The triggering of the antiviral state could occur when the interferon attaches to a surface receptor, as for the cAMP mediated effects of insulin (Cuatrecasas 1974), or may require transport of some part or all of the interferon molecule to the inside of the cell, as with the polypeptide toxins of diphtheria (Gill and Dinis 1971) and ricin (Olsnes and Pihl 1973). The former possibility requires a "second messenger", but the latter allows for interferon being directly involved in blocking virus replication. An analogous problem has already been met in the case of the effects caused by dsRNA (section 1.2).

#### Interferon binding

Early experiments suggested that little interferon bound to cells (Buckler et al 1966), but these experiments relied on detecting small reductions in a large amount of applied interferon, and so were not very sensitive. Circumstantial evidence for binding was provided by the observation that cells, treated with interferon in the cold, could be washed repeatedly before warming, and yet still be resistant to virus infection (Friedman 1967). The cold reaction was assumed to represent binding because trypsin treatment, after removal of excess interferon, was able to prevent the antiviral state developing, and the degree of resistance depended on the interferon concentration present in the temperature - independent step (Friedman 1967).

Recovery of applied interferon from cells has enabled binding to be demonstrated directly. Stewart et al (1972d) found it was possible to detect interferon in sonicates of treated mouse cells, providing large numbers of cells were used. Even though no decrease in interferon in the

medium was found (Buckler et al 1966), as much as 30% bound to the cells. The recovered activity had the characteristics of interferon. The amount bound varied according to the cell type, more sensitive cells binding more at the saturation doses used (Stewart et al 1972d). This binding was temperature dependant, implying that exposure to interferon may induce a binding or transport system normally present in limiting quantities (Sheaff and Stewart 1969a,b and c).

Using human amnion cells, Berman and Vilcek (1974) found that interferon uptake reached the same saturation level whatever the temperature, but the rate of uptake was lower in the cold. Most of the bound interferon was in a low speed pellet containing membranes, and some of this was released into the medium at 37°C but not at 4°C. Stewart (1975) has <sup>described</sup> ~~decided~~ a similar phenomenon with L929 cells, in which interferon, bound rapidly in the cold, was partly released and partly transported into a subcellular fraction upon warming.

It thus seems that the step which can proceed, albeit more slowly, in the cold represents superficial binding. This is "specific" in that heterologous interferon does not bind, but possibly some binding is to receptors other than the one involved in the antiviral state. The temperature-dependant step involves some of the interferon being lost from the cells, whilst the remainder becomes trypsin resistant and is found in a distinct subcellular fraction containing large organelles and membranes. Whether the antiviral state is induced by the cell surface interferon which is released, or by the interferon which is transported to a trypsin-resistant site, cannot be determined from these results, although the demonstration that interferon needs to be externalised by a dsRNA-treated cell before that cell develops an antiviral state (Vengris et al 1975) implies that cell surface interferon is responsible.

Various workers have attempted to immobilise interferons to test whether cell surface interferon can induce the antiviral state (Ankel et al 1973, Knight 1974b). Mouse interferon covalently bound to Sepharose beads

can protect L929 cells against virus challenge, provided direct cell to bead contact is allowed (Ankel et al 1973). Very little soluble interferon could be detected under these conditions, and the beads lost no activity upon serial transfer. However, these controls cannot exclude the possibility that locally high concentrations of free interferon may accumulate close to the cells, and may be responsible for inducing the antiviral state.

An alternative approach is to characterise the cell surface receptor. Various experiments have suggested that hydrophobic forces are important in the binding of interferons to such substrates as albumin (Davey et al 1974), concanavalin A (Davey et al 1974) and hydrocarbons (Davey et al 1975). Hydrophobic binding could be important in the cell surface interaction, with interferon recognising a lipid such as vitamin A (Blalock and Gifford 1975) or a specific ganglioside (Besancon and Ankel 1974a). The ganglioside binding is specific for a certain ganglioside and a given interferon, so could be important for the species specificity of interferons. The interferon probably recognises the carbohydrate moiety (Besancon and Ankel 1974a), which is the part of the molecule exposed at the membrane surface (Cuatrecasas 1974).

The relevance of ganglioside binding to the antiviral state was recently demonstrated when ganglioside GM<sub>2</sub> deficient mouse fibroblast clones were found to be relatively insensitive to mouse interferon. Extra GM<sub>2</sub> added to the medium was incorporated into the cells, and sensitivity to interferon was increased (Vengris et al 1976). The binding of cholera toxin, which binds to gangliosides (van Heyningen 1974), and thyrotropin to cells is inhibited by interferon, although the binding of other hormones is not. Both compounds diminish the antiviral effect of interferon, possibly by competing for binding sites (D. Kohn, personal communication 1976).

These results need to be interpreted with caution, however, until the role of gangliosides in the cell membrane and their interaction with other

lipids is understood (van Heyningen 1974). The lack of specificity of human fibroblast interferon for binding to different gangliosides (Vengris et al 1976) means that this mechanism may only be relevant for mouse interferon.

The nature of the receptor has also been investigated by treating the cell surface with enzymes. For example, phospholipase C, added before interferon, prevents the antiviral effect developing (Friedman 1967). Phytohemagglutinin can block the receptor in mouse cells, and sensitivity to interferon can be restored by absorbing the phytohemagglutinin onto fetuin (Besancon and Ankel 1974b). These experiments, and similar ones, imply the involvement of lipid and carbohydrate in the binding, but could be due to non-specific effects.

A new approach to the nature of the receptor came with the application of somatic cell genetics to the analysis of the chromosomes involved in interferon induction (reviewed by Creaghan et al 1975) and action. By making use of cell hybrid clones, phenotypic characters can be correlated to the presence of certain chromosomes. Because of the restricted species specificity of interferons, development of the antiviral state in response to an interferon active on one of the parental lines implies the presence of all gene products necessary for responding to that interferon. Any gene products which are not species specific could presumably be provided by genes from either parent.

Early work suggested that monkey genes for responding to and producing interferon were not on the same chromosome (Cassingena et al 1971). When more clones were surveyed, the antiviral genes were allocated to a monkey chromosome analogous to human chromosome G21 (Chany et al 1973). By surveying mouse x human clones, Tan et al (1973) confirmed the G21 location.

Tan and colleagues (Tan 1975; Tan et al 1975) then showed that the sensitivity of human fibroblast mutant lines was related to dosage of chromosome 21. Monosomic, disomic and trisomic lines from a range of donors were studied, and the dose of interferon required to protect 50%



of the cells was found to decrease as the number of chromosome 21 copies increased. Chany et al (1975) looked at triploid, as well as trisomic, lines and found that some other chromosomes reduced sensitivity to interferon, so that **triploid** lines were equally sensitive as diploid. Chromosome E16 was tentatively identified as carrying genes regulating or antagonising G21 genes.

As the species-specific events in the antiviral effect must include the recognition of interferon by the cell, but need not necessarily include any subsequent steps, chromosome G21 presumably carries at least those genes for the cell surface receptor or inducible transport system (Chany et al 1973; Sheaff and Stewart 1969a,b and c).

This has recently been corroborated by the demonstration that G21 codes for some cell surface proteins (Revel et al 1976). Antisera raised to surface antigens of hybrids carrying only three human chromosomes (G21 and two others) inhibit interferon **action** in human fibroblasts. The inhibitory antibodies can be adsorbed onto clones carrying G21, but not onto clones carrying the other two chromosomes. However, it seems odd that the antiserum inhibited the antiviral effect when added after interferon treatment, and it may inhibit a step after interferon binding.

Metabolic requirements for development of the antiviral state

Following the initial interaction of interferon with the cell, a second temperature-dependant step is required for the development of an antiviral state. This step can occur very rapidly (Dianzani and Baron 1975), although not so rapidly as to preclude de novo RNA and protein synthesis (Palmiter 1973), and has been variously interpreted as the induction of a new uptake system (Sheaff and Stewart 1969a,b and c; Chany et al 1973), active transport of the interferon to the interior of the cell (Stewart et al 1972d), chemical processing of the interferon (Kosower and Kosower 1975), or the synthesis of an "antiviral protein" (Levine 1964; Marcus and Salb 1966; Chany et al 1971). The concept of an antiviral protein was introduced

in the light of studies using metabolic inhibitors - it was postulated to be induced by interferon treatment, and to mediate the intracellular actions of interferon.

The development of the antiviral state is prevented by a range of inhibitors including actinomycin D, cycloheximide, puromycin, actinomycin D p-fluorophenylalanine (see de Clercq and Stewart 1973) and cordycepin (Radke et al 1974). However, under particular conditions actinomycin D may have no effect or may even increase the antiviral effect (Chany et al 1971). A similar situation is found with the "superinduction" of interferon (Tan et al 1970; Vilcek and Ng 1971), where it is attributed to the presence of a negative control system (Tan et al 1970; Vilcek and Ng 1971). In this, de novo synthesis of interferon (Walters et al 1967, de Clercq and Merigan 1970) is inhibited by the product of a second gene, which is induced by interferon. Such a system could also apply for interferon action (Chany et al 1971).

The classical system for superinduction of a gene product is the hormonal induction of tyrosine aminotransferase (TAT) in liver cells (Tomkins et al 1969). Tomkins et al (1972) have since reviewed 38 different metabolic functions which are stimulated by actinomycin D, and 15 stimulated by a range of other inhibitors. However, other groups have pointed out that actinomycin D can have many side effects (e.g. on turnover of mRNA, Singer and Penman 1972; and on initiation of protein synthesis, Schwartz 1973), and Tomkin's group have now reevaluated superinduction theory in view of these effects (Steinberg et al 1975). Protein synthesis inhibitors also cause secondary changes in the cell, including a stringent type response involving changes in initiation of protein synthesis (Reichman and Penman 1973) and in rRNA synthesis rates and nucleotide triphosphate pools (Grummt and Grummt 1976). Interpretation of superinduction, of either interferon itself or the antiviral state, as involving regulation of the expression of certain gene products (Tan et al 1970; Chany et al 1971) must thus be treated cautiously.

In view of the problems with metabolic inhibitors, nuclear involvement is perhaps better demonstrated by enucleation. Radke et al (1974) showed that enucleation at the time of interferon treatment prevented the antiviral state developing, whereas interferon added before enucleation can inhibit virus growth (Young et al 1975). This implies that the antiviral state does involve the nucleus, although functions other than transcription may be important. Even if new gene products are required, they may be necessary for interferon uptake (Chany et al 1973) rather than directly inhibiting virus replication.

#### The nature of the block in virus replication

Cells in the antiviral state do allow virus penetration and uncoating, the block in replication being at some stage in the synthesis of viral RNA or protein, or at maturation (early evidence summarised by Son nabend and Friedman 1973). Different viruses differ in their relative sensitivity to interferon according to the cells used (Stewart et al 1969), and it is possible that interferon causes different lesions according to the virus/cell system under investigation. This section will consider only the alphaviruses, vaccinia virus and SV40, and experiments using cell-free translation systems will be presented later (section 1.4).

For the alphaviruses, the earliest step which is blocked is the translation of the input genomic, (+) RNA to produce the polymerase. Mecs et al (1967) showed that all viral RNA synthesis was inhibited, and Friedman et al (1967) showed that this included primary transcription. The polymerase contains virus polypeptides encoded by the non-structural region of the genome (H. Brzeski, J.P. Clewley, J.C.S. Clegg, and S.I.T. Kennedy, personal communication 1975). The inhibition of primary transcription could thus result from either blocked translation of the genomic RNA, or inhibition of the polymerase activity. In vitro polymerase assays suggested that polymerase synthesis, but not function, was inhibited (Son nabend et al 1967), and the synthesis of some early non-structural proteins is reduced by interferon (Friedman 1968).

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Vaccinia virus, a DNA virus which replicates in the cytoplasm, is also unable to replicate in interferon-treated cells, probably because of a block in translation. After "first-stage uncoating", primary transcription of some of the DNA occurs using a virion polymerase. Complete uncoating requires protein synthesis (Magee et al 1968). Interferon treatment does not prevent the early mRNA synthesis, but does prevent the incorporation of this RNA into polyribosomes (Joklik and Merigan 1966, Jungwirth et al 1972, Metz and Esteban 1972). Second stage uncoating does not occur (Magee et al 1968) allowing increased production of the early mRNA (Jungwirth et al 1972). The lesion in translation appears to be at initiation, at a step prior to 40S subunit binding (Metz et al 1975), but elongation may also be affected (Metz et al 1975).

It thus seems that for these two types of cytoplasmic viruses, the lesion is at translation, rather than at RNA or DNA dependant transcription. Work with rhabdoviruses, and other (-) RNA viruses, has suggested that primary transcription is the target (Marcus et al 1971), but this relies on distinguishing protein synthesis dependant, from protein synthesis independant, transcription. More recent work suggests that primary transcription is unaffected, but translation is the target (Repik et al 1974).

SV40 is a DNA virus which requires some nuclear functions for its replication, and which can be integrated into the host cell DNA. Oxman et al (1967a) showed that SV40 T-antigen synthesis was sensitive to interferon in permissive, but not in transformed, cells. Both cell types were resistant to VSV infection, and both contained all the information required to make infectious progeny SV40. It was possible that the difference in sensitivity resulted from integration of the viral DNA into the host genome. As a model for integration, SV40 x adenovirus hybrids, containing SV40 information covalently attached within an adenovirus genome, were used (Oxman et al 1967b). SV40 T-antigen expression during lytic

infection by these hybrids showed the resistance to interferon typical of the adenovirus, but not the SV40, parent. These results can be explained either by supposing that SV40, integrated into host or adenovirus DNA, becomes subject to host or adenovirus transcriptional control, or, if the transcribed RNA contains SV40 sequences covalently linked to host or adenovirus sequences, by control at the level of translation.

Hybridisation showed that 27% of the SV40 RNA was indeed covalently linked to adenovirus sequences, so could escape SV40-specific translational control (Oxman et al 1974). However, when SV40 sequences were quantitated in infected cells, mRNA synthesis was found to be inhibited in cells infected with SV40, but not in cells infected with the hybrid (Oxman et al 1974). Viral transcription does not seem to require translation, and mRNA degradation is unaltered (Metz et al 1976), suggesting a primary lesion at transcription. The same conclusion is reached using cell free transcription systems (D.H.Metz, personal communication 1975). There is now evidence that the primary defect is at uncoating (Yamamoto et al 1975).

On the other hand, interferon treatment after, rather than before, infection, can prevent SV40 translation without affecting transcription (M. Revel, personal communication 1976). This is in agreement with microinjection experiments, in which SV40 T-antigen formation was detected in cells injected with SV40 cRNA, when translation was inhibited if the cells were pretreated with interferon (Graessmann et al 1974).

In conclusion, this in vivo evidence suggests that interferon inhibits viral translation in alphavirus, vaccinia virus, and SV40 infected cells, and perhaps also DNA-dependant RNA transcription in SV40 infected cells. Reports of effects on RNA-dependant RNA transcription need to be interpreted with caution (Repik et al 1974).

#### Effects of interferon on virus-induced changes in host cell functions

This section will describe the effect of interferon on the changes in host cells observed after infection with vaccinia virus and VSV; the action of interferon on uninfected cells is considered elsewhere (section 1.5). Two situations are possible. If the host function is controlled by an

intermediate in virus replication before the interferon-sensitive step, the virus may induce the change even though complete replication is prevented. On the other hand, the change will be sensitive to interferon if it is triggered by an intermediate whose formation is blocked by interferon treatment.

For vaccinia virus in L929 cells, shut off of host protein synthesis is rapid (Metz and Esteban 1972), and occurs even when virus replication is prevented by interferon (Joklik and Merigan 1966, Metz and Esteban 1972). The inhibition may actually be enhanced in interferon-treated cells (M. Suh, personal communication 1975). Shut off could be caused by the input virion components, because UV-irradiated virus is inhibitory (Moss 1968) and inhibition still occurs in the presence of actinomycin D; although there are some virus functions which are resistant to both these treatments (Rosemund-Hornbeak and Moss 1975). Vaccinia cores can inhibit reticulocyte lysate protein synthesis with the characteristics of dsRNA (H.R.B. Pelham, personal communication 1976).

High multiplicity infection with vaccinia normally causes a late cytopathic effect, which is dependant on RNA and protein synthesis (Bablanian 1970), although it is more resistant than virus multiplication to the effects of metabolic inhibitors (Bablanian 1968, 1970). If the cells were interferon treated, the cytopathic effect occurs earlier and more extensively (Joklik and Merigan 1966). It is still sensitive to inhibitors (Horak et al 1971), and may result from host cell shut off (Metz and Esteban 1972; M. Suh, personal communication 1975) or from the failure to control early mRNA synthesis (Jungwirth et al 1972).

The observation of enhanced cytopathic effects in interferon-treated cells lead Joklik and Merigan (1966) to propose a "suicide theory" for interferon action. By causing cell death, or complete cessation of a vital function such as protein synthesis, interferon could indirectly inhibit virus replication. Although this may apply for the particular case of vaccinia virus in L929 cells, it is clearly not true for other systems.

Perhaps the best demonstration that the block in virus translation is selective and not total is provided by SV40, which does not inhibit host cell protein synthesis at all. Application of interferon to SV40 infected cells prevents viral but not host translation (M.Revel, personal communication 76)

Interferon is unable to prevent VSV-induced protein synthesis inhibition (Yamazaki and Wagner 1970), which may be caused by input virion components (Baxt and Bablanian 1976). On the other hand, interferon can prevent virus cytopathic effects providing that the input multiplicity is low (Yamazaki and Wagner 1970). It has been suggested that low multiplicity cytopathic effects are due to an early product of virus replication, but at high multiplicity they are due to "killer particles" in the virus stock (Marcus and Sekellick 1974). The early product may be virus dsRNA (Marcus and Sekellick 1975) whose formation could be partly inhibited by interferon (Repik et al 1974).

Yet other cases are known where interferon, at concentrations sufficient to inhibit virus replication, does not prevent either protein synthesis inhibition or the cytopathic effect (Gauntt and Lockart 1968; Haase et al 1969; Levy 1964). RNA synthesis inhibition may also be resistant (Haase et al 1969; Levy et al 1966). Clearly, a full explanation of these effects will only be possible when the lesion in virus replication is fully characterised and the causes of host cell macromolecular synthesis inhibition and cytopathic effects are known.



#### 1.4. CELL-FREE TRANSLATION SYSTEMS FROM INTERFERON-TREATED CELLS

Interferon can inhibit viral replication by inducing a block in viral translation. This has been shown for alphaviruses and vaccinia virus (see section 1.3) and is probably also true for VSV and influenza virus (Repik et al 1974). The replication of SV40 may also be inhibited at this level (Graessmann et al 1974; M. Revel, personal communication, 1976).

Various workers have shown changes in the protein synthetic machinery of uninfected cells treated with interferon, even though the overall pattern of macromolecular synthesis is not altered (Levy and Merigan 1966). For example, Levy and Riley (1973) reported that interferon caused a slight shift in the molecular weight distribution of polyA(+)mRNA, without affecting rRNA or HnRNA. The molecular weights were slightly higher in interferon-treated cells. Viral RNA is not modified in interferon-treated cells (Klesel et al 1974), so the change in host mRNA may allow the cell to discriminate against viral RNA.

tRNA may also be modified in interferon-treated cells (Truden et al 1967; Sen et al 1976; F.L. Riley and H.B. Levy, personal communication 1976). Alteration of the balance between different isoaccepting tRNAs is probably important in the control of protein synthesis in cells (Smith 1975, Sueoka 1966), and may account for the cytolytic response of interferon-treated cells to arginine starvation (Lee and Rozee 1975).

Ng et al (1968) found that interferon promoted the synthesis of some ribosome-associated proteins, but these observations have not been repeated (E.M. Martin, I.M. Kerr, and J. Sonabend, personal communication 1968).

A more direct approach to the problem is to study the activity of cell-free protein synthesising systems on added and endogenous mRNA. Once changes in function are found, they can be correlated with changes in the structure of the machinery in a meaningful way. However, early work was unsatisfactory because knowledge of the mechanics of protein synthesis was inadequate and crude interferon was used. Marcus and Salb (1966) assumed that protein synthesis was occurring when "polyribosomes", formed by

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binding ribosomes to mRNA in the cold, broke down during incubation. The ionic conditions are now realised not to be suitable for polyribosome formation (Schreier and Staehelin 1973b), and the disaggregation observed on warming could be due to ribonuclease activity. Kerr et al (1970 and Kerr 1971) could not repeat the observations.

Using more defined systems, some effects have been reproducibly observed in extracts of interferon-treated cells (for example, Gupta et al 1973, Gupta et al 1974a, Falcoff et al 1972, and Content et al 1974), although other groups have failed to observe effects unless the cells had also been infected (for example, Friedman et al 1972a), or even under any conditions at all (G. Hiller, personal communication 1975). The possibility that infection is required for full expression of the translational defect has led to a basic dichotomy in experimental approach and theory.

On the one hand, P. Lengyel, R. Falcoff, and M. Revel and colleagues have concentrated on characterising the defects they find in systems from interferon-treated but uninfected cells. To resemble the in vivo situation, these in vitro systems are expected to discriminate between viral and host mRNAs.

On the other hand, I. Kerr's group has compared systems from interferon-treated infected cells, and untreated infected cells. They hypothesize that virus infection triggers a defect in translation in interferon-treated cells, not present in uninfected cells. Such systems need not discriminate between virus and host mRNAs.

These two lines of evidence will be considered in turn, before discussing recent developments concerning nuclease activity and mRNA modification in interferon-treated cell extracts.

#### Cell-free systems from uninfected cells

Preincubated and gel-filtered cell-free systems, from mouse ascites or L929 cells, show a low background of endogenous synthesis and can utilise

added EMC, mengo, reo or a variety of other virus and cell mRNAs (Gupta et al 1973, 1974a, Samuel and Joklik 1974, Falcoff et al 1972). These systems show lower efficiency when made from interferon-treated cells, but this effect is in all cases most readily seen after preincubation and gel-filtration. The significance of this, and the choice of mRNA, will become apparent later.

The ascites system of Gupta et al (1973) translates EMC mRNA with high efficiency. They found a component in the post-ribosomal supernatant of interferon-treated cells, which inhibited protein synthesis in control cell extracts. However, when non-preincubated extracts were fractionated and tested for inhibitory activity, the inhibitor was found on the ribosomes. The inhibitor thus moved off the ribosomes during preincubation (Gupta et al 1973). It seemed that elongation, rather than initiation, of polypeptide chains was inhibited, because polysomes from control extracts could not be utilised by interferon extracts (Gupta et al 1974b), although there might have been a secondary effect on initiation.

The same group, using L929 cells and reovirus mRNAs (Graziadei et al 1973a), found a similar inhibitor in interferon-treated cell extracts (Gupta et al 1974a). This confirmed the findings of Falcoff et al (1972) using mengovirus or globin mRNAs. Unlike the ascites cell inhibitor, the L929 cell inhibitor was firmly bound to the ribosomes and was not dominant (Falcoff et al 1972). Elongation was again the primary site of inhibition (Content et al 1974), but a secondary effect on initiation was also characterised (Content et al 1975).

None of these results showed any discrimination between viral or host mRNA, and the only RNA which escaped the block was poly rU, which is normally assayed under conditions where abnormal initiation occurs (Falcoff et al 1973, Gupta et al 1974a, Gupta et al 1973). However, when tRNA was added, the

activity of interferon-treated extracts could be restored to control levels (Gupta et al 1974c, Content et al 1974), and this tRNA dependance was critical on the ionic conditions of the assay (Gupta et al 1973, 1974b). Different fractions of tRNA restored the translation of globin and mengovirus mRNA with different efficiency (Content et al 1974, 1975). Thus it was possible to create mRNA discriminating systems by the selective addition of tRNA, and it was possible that these tRNAs were artefactually lost during preparation of the system.

Charging of leucine, lysine, and serine tRNAs is defective in interferon-treated extracts (Gupta et al 1974b, Sen et al 1976), and the translation of poly(rU,rC), which codes for polyleucine, is strongly inhibited (Falcoff et al 1976). Synthesis directed by mengovirus mRNA or by poly(rU,rC) is restored by a tRNA fraction corresponding to one of the leucine accepting tRNA species (Content et al 1974, 1975, Falcoff et al 1976). Synthesis using globin mRNA is restored by a different fraction of tRNA.

These effects of interferon showed all the characteristics expected - in terms of dose-response, effects of actinomycin D, and persistence of the defect after interferon removal (Falcoff et al 1973) - the translation defect always paralleling the antiviral effect. It seemed that interferon could act by causing a defect in either the tRNA or the charging enzymes, such that translation of virus mRNA was impaired whilst host translation continued, so inhibiting virus replication. However, there are problems in this hypothesis.

If the tRNA were inactivated by interferon treatment, tRNA extracted from interferon-treated cells should be less effective at reversing the translation block than tRNA from control cells. This is not the case (Content et al 1974, Sen et al 1976). On the other hand, if the tRNA charging enzymes were inactivated, addition of purified charging enzymes should overcome the block, but they do not (Sen et al 1976).

This was resolved by the finding that tRNA from interferon-treated extracts was less effective than tRNA from control extracts, provided that the extracts were gel-filtered (Sen et al 1976). This functional inactivation was prevented by the addition of a variety of low molecular weight compounds, including sodium ions, calcium ions, and polyamines. Polyamines are known to be important in other systems for efficient translation (Atkins et al 1975), and to stabilise RNA (Kyner et al 1973, Igarashi et al 1974).

Lengyel and colleagues (Sen et al 1976) have concluded that the effects observed in preincubated and gel-filtered extracts are not operative in the intact cell. There are some changes observable in non-preincubated extracts (Sen et al 1976) and these are not reversed by tRNA and operate by a different mechanism. The fact remains that one action of interferon is to render certain tRNAs more labile under certain conditions, and this could be due to activation of a nuclease or alteration in tRNA structure.

It is possible that the report of Samuel and Joklik (1974), that a message-discriminating system could be prepared from interferon-treated ascites cells, is also due to a tRNA effect. Preincubation was required, and the incubations routinely contained added tRNA. Their finding that inhibition correlated with the appearance of a 48000 dalton polypeptide in the ribosomal salt wash fraction may thus indicate the molecular basis for the tRNA effect. Other explanations for message discrimination in their system will be outlined below.

#### Cell-free systems from infected cells

The initial failure to find any significant difference between non-preincubated cell-free systems from interferon-treated and control cells (Friedman et al 1972a, Falcoff et al 1972) led Kerr's group to study systems from interferon-treated, virus-infected cells (Friedman et al 1972a,b).

The experimental approach was to treat the cell suspension with a low dose of partly-purified interferon, infect with vaccinia virus or EMC virus, and then make cell-free extracts at a time when viral inhibition of host

protein synthesis was well advanced (Kerr et al 1973). The systems were neither preincubated nor gel-filtered, but added EMC mRNA was translated efficiently and gave discrete products (Kerr and Martin 1971, Esteban and Kerr 1974, Smith 1973).

When interferon and control cells were infected, and extracts made, there was some inhibition of endogenous translation, and pronounced inhibition of EMC mRNA translation. No effects could be detected in uninfected extracts (Friedman et al 1972a,b). The difference was induced by a trypsin-sensitive component of the interferon preparation, and was evident under a wide range of assay conditions. The inhibition was not mRNA specific, and did not involve mRNA degradation (Friedman et al 1972b). It was not overcome by added tRNA (I.M. Kerr, personal communication 1975).

The lesion was due to an inhibitor which was present mainly in the post-ribosomal supernatant (Kerr et al 1974a), and was destroyed by preincubation (Friedman et al 1972b), in contrast to the tRNA defect which requires preincubation (Falcoff et al 1972). Analysis of the lesion was difficult because EMC mRNA codes for a wide range of polypeptides, produced both by post-translational cleavage and by premature termination of polypeptide chains (Smith 1973, Esteban and Kerr 1974). However, use of an N-formylated initiator tRNA showed that initiation is the primary site of the block (M.Esteban, personal communication 1973; Kerr et al 1974a) although there is also some inhibition of elongation. The precise nature of the inhibition is still uncertain, but it may be an effect on the met tRNA<sub>f</sub> binding reaction, as shown for preincubated extracts by Content et al (1975)

It thus seemed that infection with a virus was able to trigger the formation of an inhibitor in interferon-treated cells, so Kerr et al (1973) investigated the possibility that other treatments which lowered host cell protein synthesis might also trigger the inhibition. Starving cells of aminoacids briefly before making cell-free extracts produced systems which were rather less active than controls (Kerr et al 1966), but were still able

to utilise added mRNA (Kerr et al 1973). Interferon treatment, followed by starvation, gave systems having the same endogenous synthesis as starved control cells, but which were impaired in the translation of added mRNA (Kerr et al 1973).

More recent work has suggested a candidate for the "trigger" in virus-infected cells. Kerr et al (1974b) showed that extracts from interferon-treated, but not infected cells were inhibited by low concentrations of dsRNA, whereas dsRNA had little effect on control extracts. The addition of dsRNA to the cell-free incubations was thus equivalent to infecting the cells with virus. Either viral or synthetic dsRNA was effective, but DNA and ssRNA were not. It remains to be seen whether the mechanism of this inhibition is the same as that occurring in the virus-infected systems; if so, it would seem likely that dsRNA produced as an intermediate in virus replication (Colby and Duesberg 1969) could trigger the inhibition of translation.

The dsRNA effect has now been repeated (Sen et al 1976), and provides explanations for a variety of observations in the literature. The ability of Gupta et al (1974a) to find inhibition in extracts of interferon-treated, uninfected cells, may reflect their use of mRNA preparations synthesised in vitro from reovirus cores (Graziadei et al 1973a), because these mRNAs contain dsRNA (Brown et al 1976). The microinjection experiments of Graessmame et al (1974), in which SV40 cRNA translation in intact cells was shown to be interferon sensitive, used cRNA preparations containing about 20% self-annealing material (Peterson et al 1972). On the other hand, Samuel and Joklik (1974) used salt precipitation to purify their preparations of in vitro synthesised reovirus and vaccinia virus mRNAs, so gross contamination with dsRNA is unlikely.

#### Other effects relevant to cell-free translation systems

Ribonuclease activity can inhibit cell-free protein synthesis by degrading mRNA or interfering with the protein synthesis machinery. Reports that mouse interferon itself has endonuclease activity (Graziadei et al 1973b) unfortunately cannot be substantiated until repeated with purified preparations (Knight 1975). However, there is also evidence that cellular nuclease activity may be stimulated



by interferon treatment.

Marcus et al (1971,1975) investigated the replication of VSV in interferon-treated chick cells. It appears that viral RNA synthesis, using a virion enzyme, is not inhibited (Marcus et al 1971), but that interferon treatment induces a membrane-bound ribonuclease able to degrade VSV mRNA (Marcus et al 1975). The alkaline pH optimum and magnesium dependence suggested this was a different activity from that found in untreated cells (Marcus et al 1975). The significance of this activity is diminished by the observations that protein synthesis is probably the target for interferon in VSV-infected chick and mammalian cells (Repik et al 1974), and the absence of a similar nuclease in interferon-treated monkey cells (Maenner and Brandner 1976).

Brown et al (1976) have reported the existence of a dsRNA-activated mRNA-specific ribonuclease in extracts from interferon-treated ascites cells. This activity was present in extracts which had not been preincubated or gel-filtered, and was readily detected using sedimentation analysis to examine the fate of radioactive mRNA. mRNA which had been freed of dsRNA by salt precipitation was not degraded, unless dsRNA was added as well (Brown et al 1976). It is unlikely that the chick cell nuclease is also dsRNA-activated because it was active on L cell polyA(+)mRNA, which is unlikely to contain much dsRNA contamination (Marcus et al 1975). This work has recently been extended to show a requirement for ATP, as well as dsRNA, in activating the nuclease (P. Lengyel, personal communication 1976). Once activated, the nuclease retains activity even when the dsRNA is destroyed with RNase III and ATP is removed with hexokinase and glucose. The enzyme acts as an endonuclease on long, rather than short, mRNAs, and does not degrade rRNA. Degradation is not dependent on methylation of the mRNA or on protein synthesis, and so is independent of the other effects of interferon and dsRNA.

The capping and methylation of mRNA (synthesis of a  $m^7GpppX^m$ ... structure at the 5' end) has recently been the subject of much investigation.

Many eukaryotic mRNAs are capped and methylated (some are listed in Furuichi et al 1975) but some are not (for example, poliovirus mRNA, Hewlett et al 1976). The importance of the modification for translation, once thought to be absolute (Both et al 1975, Muthukrishnan et al 1975), is now realised to vary according to the cell-free system used (Canaani et al 1976), and perhaps even with the ionic conditions (A.E. Smith personal communication 1976).

The methylation of reovirus mRNAs probably requires virion enzymes (reviewed by Shatkin and Both 1976), although cellular enzymes could also be involved. Sen et al (1975) reported that the methylation of reo mRNA by cellular enzymes was defective in interferon-treated, but not control, cell-free extracts. This work has recently been developed (P. Lengyel, personal communication 1976) to show that the methylation of mRNA de novo synthesised by reovirus cores is also inhibited in interferon-treated extracts. The inhibition is due to a macromolecular inhibitor and not to degradation of either the methyl donor, S-adenosyl methionine (SAM), or the mRNA substrate. The inhibitor is labile and destroyed by preincubation.

The significance of impaired methylation in vivo has not yet been demonstrated, but it is interesting that VSV mRNA methylation in a coupled cell-free transcription/translation system from chick cells is probably not inhibited (A. Ball, personal communication 1975). However, the effect is useful for explaining published results using protein synthesis systems, and the observation that certain viral mRNAs are translated poorly in interferon-treated cell extracts may partly be due to their preparation. In vitro vaccinia and reo mRNAs are not expected to be methylated unless SAM is present in the reaction mixture (Ensinger et al 1975, Shatkin and Both 1976), so the mRNAs used by Gupta et al (1974a) and Samuel and Joklik (1974) were probably not methylated. If methylation is defective in cell-free extracts from interferon-treated cells (Sen et al 1975a) and if translation depends on methylation (Canaani et al 1976), then defective translation of reovirus and vaccinia virus mRNA is not surprising.

A final complication to the story is found when mitochondrial protein synthesis is examined (Kortsaris et al 1976). Isolated mitochondria were found to synthesise protein and to be stimulated by added EMC virus or globin mRNA. Synthesis was probably occurring on 70S ribosomes (i.e. was chloramphenicol sensitive and cycloheximide resistant) and some of the added mRNA entered the mitochondria, but the products were not characterised and may not be meaningful. The reduced activity of mitochondria from interferon-treated cells is thus of dubious significance particularly as the effects on energy generation and precursor transport were not determined (Kortsaris et al 1976).

### Conclusions

There are several differences between in vitro systems made from interferon-treated and control cells. Certain species of tRNA are more rapidly inactivated after gel-filtration (Content et al 1975; Sen et al 1976); and this prevents elongation of nascent polypeptide chains, with a secondary effect on initiation (Content et al 1975). A macromolecular inhibitor of mRNA methylation, by both virus and cytoplasmic enzymes, is generated (Lengyel et al 1976), and a dsRNA-activated endonuclease (Brown et al 1976), and perhaps other nucleases (Marcus et al 1975), are produced. Finally, an inhibitor of protein synthesis, probably acting on initiation (Kerr et al 1974a), is generated if dsRNA is added to the extract (Kerr et al 1974b), or if the extract is made from infected cells (Friedman et al 1972b).

All these effects are dependant on treatment of the cells with homologous interferon, and, when tested, the effects parallel the antiviral activity. In no case is purified interferon, added to the cell free system, able to reproduce the effect on interferon treatment of the intact cells. However, this does not exclude the possibility that suitably modified, intracellular, interferon is mediating the in vitro actions.

It is still unclear how the in vitro effects relate to the in vivo effects - which are causal, which are coincidental, and which have no relevance at all in the intact cell. As described above, some of the apparent effects seen in vitro are critically dependant on cell type, treatment of the cell-free system, source of mRNA, and choice of ionic conditions for assay. Even so, it is possible that the variety of in vitro actions are related to the variety of antiviral and non-antiviral actions of interferon seen in intact cells. Thus Kerr et al (1974b) suggested that the dsRNA-dependant inhibition of protein synthesis might relate to the cytotoxic effect of dsRNA (Stewart et al 1972a), and R. Falcoff (personal communication 1974) described a reduction in in vitro protein synthesis activity at high doses of interferon, which might be related to cell growth inhibition (Paucker et al 1962).

It is interesting that dsRNA, which is an interferon inducer and is more toxic for interferon-treated than control cells, is required for at least two of in vitro actions (Kerr et al 1974b, Brown et al 1976). The possibility that dsRNA is the key molecule by which a cell senses virus infection, and is thus the mediator of a wide range of virus/cell interactions, is discussed in a later section.

### 1.5. NON-ANTIVIRAL ACTIONS OF INTERFERON

Only in recent years have effects of interferons on uninfected cells become apparent (Levy and Merigan 1966; de Clercq and Stewart 1973). Previously such effects had been attributed to contaminants in the interferon preparations, or had not been reproducible in other laboratories. It now seems that partially-purified interferon preparations can produce a number of different physiological effects in whole animals and in lymphocyte cultures (reviewed by de Clercq and Stewart 1973, and by Carter and de Clercq 1974) and in tissue culture. The component of the interferon preparation responsible for the non-antiviral effects is the same as that responsible for the antiviral effect, in that it is trypsin sensitive, of high molecular weight, stable to low pH, and of restricted species specificity.

The tissue culture effects are inhibition of cell growth, blocking or priming of interferon induction, enhancement of the toxicity of dsRNA, and alteration of the cellular response to some other compounds. Each activity will be described individually before considering two questions. Firstly, in what ways do the metabolic requirements for developing the non-antiviral effects differ from the requirements for the antiviral effect? Secondly, in view of the molecular heterogeneity of interferons, is the same molecular species involved in causing all the different effects of interferons?

#### Cell growth inhibition

Interferon inhibits the growth and division of cells in suspension culture (Paucker et al 1962, Gresser et al 1970) and in monolayers (Lee et al 1972, O'Shaughnessy et al 1972). Generally it is found that the dose of interferon required is relatively high (about 100 times the antiviral dose, Paucker et al 1962), and that the doubling time is increased up to about two-fold (Paucker et al 1962). Both diploid and aneuploid cells are affected (Lee et al 1972).

The earliest changes seen in continuous culture are in the incorporation of thymidine into DNA and in the proportion of cells in S-phase; after treatment of L1210 cells with 6000 u/ml interferon, both parameters drop by about 50% in 2 hours, that is, several hours before an increase in generation time is apparent (Tovey et al 1975). In this system, the decrease in DNA synthesis corresponds to a decrease in the activity of the thymidine pool, suggesting either feedback inhibition or a primary action of interferon on transport. When synchronised cells are treated with interferon, there is a puromycin-sensitive delay at the start of S-phase suggesting that a protein inhibiting entry into S-phase may be induced (O'Shaughnessy et al 1972).

Synthesis of RNA and protein is not greatly inhibited in continuous culture (Tovey et al 1975), although protein synthesis is inhibited in batch culture (Borecky et al 1972). Neither approach is without pitfalls. The chemostat makes use of glucose limitation to control cell growth, and this could have other effects. Batch culture measurements have to be made during exponential phase growth, and culture conditions are continuously changing. The discrepancy over protein synthesis effects thus may not be significant.

The continued presence of interferon may result in the production of interferon-resistant cells (Gresser et al 1970, Katz et al 1974, Lackovic and Borecky 1976). These cells are able to synthesise RNA and grow at normal rates in the presence of interferon, and may be resistant to other actions of interferon (Gresser et al 1970, Tovey et al 1975). They may also differ in membrane properties and morphology from the parental line (Katz et al 1974). These experiments were all performed using aneuploid cell lines maintained in the presence of interferon for several passages. The high frequency with which resistant cells are found resembles the development of colchicine resistance in Chinese hamster cells (Minor and Roscoe 1975) which has been attributed to changes in karyotype affecting membrane permeability. The limited data available on the interferon-resistant

lines makes it unlikely that they are point mutations, which normally occur at very low frequency and are highly stable (Meiss and Basilico 1972).

#### Priming and blocking of interferon production

When cells are treated with interferon their response to interferon inducers may be blocked or primed.

Blocking of interferon production was first recognised with viral inducers, where production of the inducing molecule may be prevented by the antiviral state (Friedman 1966), but it can also occur with dsRNA inducers (Youngner and Hallum 1969, Stewart et al 1971b). Blocking normally occurs with high doses of interferon (greater than 1000 times the antiviral dose, Barmak and Vilcek 1973), and may be a regulatory mechanism controlling interferon production or be due to impurities.

Priming of interferon production is normally found with low doses of interferon, and can even be found in cells which exhibit blocking at high interferon doses (Friedman 1966, Barmak and Vilcek 1973, Stewart et al 1971b). Depending on cell type and inducer, priming can put up the yield of interferon dramatically; for example, in L929 cells induced with MM virus the yield is increased 400 fold (Stewart et al 1971a) and with poly rI.rC, 30-100 fold (Stewart et al 1973b).

Interferon production by primed cells occurs more rapidly (Stewart et al 1971b), and sooner becomes actinomycin D resistant (Stewart et al 1971a), than production by unprimed cells. After priming, a poor producer line of L929 cells became equally productive as a high producer line (Stewart et al 1972c). These results suggest that priming may remove a block which normally limits interferon mRNA transcription.

#### Enhancement of toxicity of dsRNA

dsRNA may be toxic to normal cells (Cordell-Stewart and Taylor 1971, 1973b), possibly because it inhibits DNA (Teng et al 1973) and protein (Cordell-Stewart and Taylor 1973a) synthesis. However, conditions can be found where low doses of dsRNA are not toxic, unless the cells have previously been treated with interferon (Stewart et al 1972a). The active

component in the interferon preparation has the characteristics of interferon (Stewart et al 1972a), and the toxic effect has even been suggested as a rapid interferon assay (Stewart et al 1972b).

Enhanced cytotoxicity is specific for dsRNA and is not due to a general increase in susceptibility to all toxic compounds or all nucleic acids (Stewart et al 1973a). Apart from dsRNA only vaccinia virus also shows interferon-enhanced toxicity (Joklik and Merigan 1966), and this may involve intracellular production of dsRNA (Colby and Duesberg 1969, Stewart et al 1973a). The molecular size requirements for a dsRNA to be toxic parallel those for induction (Stewart and de Clercq 1974), as do the metabolic and timing requirements for the development of the effect (de Clercq and de Somer 1974b). However, priming and enhanced toxicity can be separated, for example by treating with dsRNA before, as well as after, interferon treatment; a procedure which prevents primed interferon production but not the cytotoxic effect (de Clercq and de Somer 1974b).

The toxicity is rapid and extensive (Stewart et al 1972a), involving structural changes in many cellular organelles (Heremans et al 1976), but the metabolic changes caused by the dsRNA have not been identified. RNA and protein synthesis during the dsRNA treatment period are not required (de Clercq and de Somer 1974b) so that primed cells treated with dsRNA and metabolic inhibitors will die, but not produce interferon.

#### Other effects

The other non-antiviral effects of interferons have been less well characterised. They fall into two main classes; altered toxicity of compounds, and alterations in the inducibility of various enzymes.

Thus, interferon treatment is said to protect chick cells against the cytotoxic action of streptomycin (A. Yabrov, personal communication 1974) and to increase the toxicity of polycations (Katz et al 1974), concanavalin A (J. Suzuki and S. Kobayashi, personal communication 1974) and high concentrations of choline or urea (R. Ahl, personal communication 1976). Lee and Rozee (1975) have reported lysis of interferon-treated cells



in arginine-free medium, but not in media lacking other aminoacids. Addition of precursors to arginine, but not non-metabolisable analogues, prevented lysis. They suggested that this lytic effect and the antiviral effect could both result from changes in tRNA balance, although the involvement of arginine in other cell processes besides protein synthesis (for example, polyamine synthesis, Russell 1973), makes a variety of other theories possible.

Interferon can also modulate specific responses in specialised cells. One of the many effects on lymphoid cells is to inhibit the action of mitogens on spleen cells (Lindahl-Magnusson *et al* 1972). This inhibition can be increased by prior treatment with "priming" doses of interferon (Rozes *et al* 1973). Rossi *et al* (1975) showed that interferon can inhibit the DMSO induced differentiation of Friend leukemia cells, although Swetly and Ostertag (1974) disagree. Both of these effects may relate to the cell-growth inhibitory effect of interferon.

Rat hepatoma cells can be induced to synthesise tyrosine aminotransferase using dexamethasone. These cells are rather insensitive to rat interferon, making studies using partly purified, low titre, preparations difficult to interpret. The induced enzyme activity was decreased by prior interferon treatment, but 200-700 times the VSV yield reducing dose was required (Vassef *et al* 1974). Illinger *et al* (1976) met similar problems while studying the hydrocortisone induction of glycerol-6-phosphate dehydrogenase in rat glial cells. Again, impure preparations were used, at high titre. Glutamine synthetase induction by hydrocortisone in chick embryo neural retina cells is inhibited by 100-300 times the EMC inhibiting dose of chick interferon (Matsuno *et al* 1976). All these experiments involved interferon doses where growth inhibition may become important.

By contrast, increased activity of an induced enzyme has been reported (Nebert and Friedman 1973). Benzantracene induction of arylhydrocarbon hydroxylase activity in liver cells is increased by interferon pretreatment. Whether this is due to altered activity or rate of synthesis of the enzyme is unknown, but uptake of the inducer and degradation of the enzyme are unaltered (Nebert and Friedman 1973).

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### Metabolic and genetic requirements for development of non-antiviral effects

The antiviral effects of interferon require a temperature and time dependant step which may require RNA and protein synthesis, and involve the nucleus - specifically, in human cells, chromosome 21 (Dianzani and Baron 1975, Radke et al 1974, Revel et al 1976). Historically, this has been interpreted as the involvement of an "antiviral protein", although this concept is debatable (see discussion in section 1.3). The metabolic requirements for, and the chromosomes involved in, some of the non-antiviral activities of interferon have also been determined.

The requirements for developing the cell growth inhibitory effect cannot be determined directly using metabolic inhibitors, because the cells need to grow through several generations. However, O'Shaughnessy et al (1972) showed that S-phase was delayed by interferon treatment in synchronised cells, and this delay was prevented by puromycin. The suggestion that an "antiviral protein" may be involved was corroborated by Tan (1976) who showed that chromosome 21 <sup>arr</sup>confers sensitivity to the cell growth inhibitory, as well as the antiviral (Tan 1975), action of human interferon. Cells resistant to cell-growth inhibition are commonly also resistant to the antiviral effect (Gresser et al 1970, Katz et al 1974) although this is not always true (V. Lackovic and L. Borecky, personal communication 1976).

Inhibitors are also difficult to use when investigating priming and blocking, because their effects must be fully reversed during the induction and production period, to avoid inhibition or superinduction phenonema (Tan et al 1971). However, it appears that blocking requires reasonably long exposure to interferon during which RNA and protein synthesis are permitted (Stewart et al 1971b, Barmak and Vilcek 1973). On the other hand, priming occurs rapidly, even in the presence of metabolic inhibitors (Stewart et al 1971b, Barmak and Vilcek 1973, Stewart et al 1971a). Not only does priming develop rapidly, it also decays rapidly (I. Rosztoczy, personal communication 1974). No increase in interferon yield can be detected when challenged 24 hours after priming, although the antiviral effect persists

through several cell generations (Paucker and Cantell 1963).

Direct membrane involvement in priming is also suggested by the finding that insolubilised interferon can prime (Knight 1974a), although leakage from the support may occur (section 1.3).

The requirements for priming probably also apply to enhanced cytotoxicity, although the use of inhibitors is difficult because they may compound the cytopathic effect. A state which is "primed" for toxicity develops rapidly (Stewart *et al* 1971b), although perhaps not as rapidly as the state primed for interferon production (de Clercq and de Somer 1974b). There is unpublished evidence (quoted in Stewart 1975) that protein synthesis is not required.

Unlike the cell growth inhibitory and antiviral effects, neither priming nor toxicity enhancement are affected by the dosage of chromosome 21 in human cells (de Clercq *et al* 1975a). It would be interesting to know whether any other chromosomes are required. Enucleation experiments could not be performed in connection with priming, but cells could be enucleated, exposed to interferon, challenged with dsRNA, and observed for cytopathic effects.

#### Molecular requirements for non-antiviral effects

Each of the non-antiviral effects is due to a protein which co-purifies with the antiviral activity. Thus, Stewart *et al* (1973b) followed three different sources of mouse interferon over a 100 fold range of specific activities, and found good correlation of antiviral, priming, and toxicity-enhancing activities. Toxicity and priming correlate over a 1000 fold range of purity (Stewart *et al* 1972a), and very highly purified preparations show a cell growth inhibitory effect (Tovey *et al* 1975 for mouse interferon; Knight 1976 for human fibroblast interferon).

The discovery that interferons may be heterogeneous led to the question of whether one component had all activities, or whether each activity was carried on a separate component.

Heterogeneity can be shown using antisera (Berg *et al* 1975, Havel *et al* 1975, Paucker *et al* 1975), affinity columns (Janowski *et al* 1975, Davey *et al* 1976), isoelectric focussing (Dorner 1973), and denaturing

polyacrylamide gels (Stewart 1974). Some of the heterogeneity is present in the carbohydrate portion (Dorner 1973), but different polypeptides may also be found. L929 cell interferon contains between 2 (Stewart 1974) and 11 (Knight 1975) distinct components, and human leukocyte interferon has two components (Stewart and Desmyter 1975).

The different components of human interferon may be responsible for cross-reactivity in rabbit (Paucker et al 1975, Stewart and Desmyter 1975) and cat cells (W.E. Stewart II, personal communication 1975). However, the components of L929 and leukocyte interferon, separated on denaturing gels, all show the same relative titres in antiviral, priming, and toxicity assays (Stewart et al 1976).

A report that the antiviral and cell growth inhibitory activities of mouse interferon could partially be separated on non-denaturing gels (Borecky et al 1972) needs some consideration. Cell growth inhibition was measured by inhibition of protein synthesis, and the fractions which were said to contain no growth inhibitory activity (yet still had antiviral activity) rely critically on the control value for protein synthesis - raising the control value by only 15-20% would give almost perfect correlation between protein synthesis inhibition and antiviral activity! Moreover, Sułkowski and colleagues (R.F. Buffet, personal communication 1976) using high activity mouse interferon, which inhibited 3T3 cell growth by 50% at only 10 u/ml, have shown that the two components separable on an affinity column are equally inhibitory.

Albumin agarose separates human leukocyte interferon into two components (Janowski et al 1975) which may differ in anticellular activity (H. Dahl and M. Degre, personal communication 1975). Confirmation of this would be interesting, in view of the failure to separate antiviral and anticellular activities on denaturing gels (Stewart et al 1976).

In conclusion, even though different molecular species of interferons may be produced by cells derived from different tissues (Berg et al 1975,

Janowski et al 1975, Stewart et al 1974), and possibly in response to different inducers (Janowski et al 1975), it seems that each component expresses all the different activities of interferon in a given species of cell. The only differences which can be detected are seen when the antiviral activity is compared in cells from different animal species (Steward and Desmyter 1975) and may reflect differences in recognition by cell-surface receptors. The results are consistent with individual molecules of interferon inducing the whole range of different effects in the recipient cells.

### 1.6. dsRNA IN THE CONTROL OF MACROMOLECULAR SYNTHESIS

dsRNA has many biological effects: in vivo it induces interferon and causes cytopathic effects in interferon-treated cells, and in vitro it may induce an inhibitor of translation and a mRNA-specific ribonuclease. This section will discuss evidence relating to the presence of dsRNA in normal and infected cells, and the possible involvement of dsRNA in the control of protein synthesis.

#### dsRNA in uninfected cells

Small amounts of dsRNA can be detected in uninfected cells (Colby and Duesberg 1969, Harel and Montagnier 1971, Stern and Friedman 1970), and as a host background in studies on the production of viral dsRNA (Dubois 1974). Detection normally utilises ribonuclease to selectively digest contaminating ssRNA, after which dsRNA is isolated by gel filtration (Harel and Montagnier 1971). The physical studies have thus been performed on molecules from which any single-stranded regions have been removed.

The dsRNA is heterogeneous, sedimenting at about 8 S (Harel and Montagnier 1971), and is of high enough molecular weight to induce interferon when applied to cells (de Maeyer et al 1971). The high melting temperature and the base composition suggest high complementarity between the strands. When melted and annealed to cellular DNA, more than 20% of the labelled dsRNA enters hybrids, compared with less than 1% annealing in controls containing E. coli RNA or native dsRNA. This 20% was not reduced by competition with rRNA, and this evidence, coupled with the base composition and the stability of its RNA.DNA hybrid, suggested that its sequences were not related to rRNA (Harel and Montagnier 1971). Ribonuclease-resistant RNA is also synthesised in the presence of concentrations of actinomycin D adequate to fully inhibit rRNA synthesis (Stern and Friedman 1970). This would seem to exclude its production from hairpin loops in ribosomal precursor RNA, as occurs in bacteria (Dunn and Studier 1973). Instead the dsRNA may

be synthesised by an actinomycin D resistant route, either using a DNA or an RNA-directed polymerase.

Of the DNA-dependant activities present in the cell, nucleoplasmic RNA synthesis is relatively insensitive to actinomycin D, although the molecular basis for this is not well understood (Lindell 1976). This activity could be responsible for dsRNA synthesis, either as hairpin loops or by as symmetric transcription of dsDNA. Random RNA sequences are highly likely to contain inverted repeat regions able to form hairpins (Gralla and De Lisi 1974), and hairpins do indeed occur in Hn RNA (Jelinek et al 1974) where they probably correspond to transcripts from hyper-repetitious DNA. Pays (1976) has shown dsRNA in in vitro chromatin transcripts, presumably due to asymmetric transcription.

The high proportion of dsRNA which does not anneal to host cell DNA might represent sequences not found in the genome, although it could merely be due to inappropriate reaction conditions. Different sequences, or amplification of uncommon sequences, could arise if the cells contained an RNA-dependant RNA polymerase, such as the one found in reticulocytes (Downey et al 1973). This activity is fully dependant on added RNA, but not DNA, template, and is inhibited by rifamycin (which inhibits the Q $\beta$  RNA-dependant RNA polymerase) but not by rifampicin (which inhibits prokaryotic DNA-dependant RNA polymerase, and oncornavirus reverse transcriptase). Such an enzyme could produce complementary RNA, which, when deproteinised, could anneal to form dsRNA. Reticulocytes are specialised cells and are enucleate but similar enzymes have been reported in rat brain microsomes (Mikoshiba et al 1974) and on the ribosomes of avian erythrocytes (Boyd and Fitschen 1975). It is possible that the RNA dependance of these enzymes represents elongation from a 3' terminal, rather than synthesis of a complementary strand (Mikoshiba et al 1974).



There is other evidence that the cell is able to process dsRNA. Perez-Bercoff et al (1974a) have shown that mengovirus RF is infectious, and that actinomycin D and  $\alpha$ -amanatin can inhibit the early stages of infection. This may mean that the input dsRNA acts as a template for a cellular polymerase, particularly as the  $\alpha$ -amanatin sensitive cellular RNA polymerase binds to dsRNA (Perez-Bercoff et al 1974b) and the input dsRNA becomes associated with newly synthesised RNA (R. Perez-Bercoff, personal communication 1976). However, recent evidence that initiation of protein synthesis can occur on viral dsRNA (Szekely et al 1976) makes it possible to interpret these results as showing the binding of ribosomes to dsRNA followed by viral polymerase synthesis and the normal course of infection. Nothing is yet known about the antibiotic sensitivity of dsRNA-dependant translation.

dsRNA as an intermediate in virus replication

The replication of RNA viruses requires synthesis of both message sense (+) and complementary sense (-) RNA. Except in the case of the diplomona viruses, where (+) RNA synthesis occur in the input virion cores and (-) RNA synthesis occurs in minus-strand synthesising particles (Burke and Russell 1975), both strands are present in the cytoplasm. Therefore the presence of dsRNA in extracts of infected cells could mean that dsRNA exists as such in the cytoplasm, although it may arise by annealing of complementary strands during extraction.

Besides dsRNA (the replicative form, RF), extracts of infected cells usually contain a multistranded structure, the replicative intermediate (RI), thought to consist of a single RNA template strand with multiple nascent complementary strands (Levin and Friedman 1971, Bruton and Kennedy 1975). In bacterial (Billeter et al 1966, Feix et al 1967) and animal virus systems (Martin and Burke 1974, Bruton and Kennedy 1975), the RI is the main intracellular structure with the RF playing a minor role or being an artefact of phenol extraction.

The RI may contain some double-stranded regions. Two different structures have been postulated; either the template and nascent strands could be held apart by proteins, with contact only at the growing point (3' end) of the nascent strand, or each nascent strand could be hybridised to the template until displaced by the next strand. The former structure contains much less dsRNA than the latter, and corresponds to Q $\beta$  RI formed in vitro (Feix et al 1967) and R17 RI in vivo (Thach and Thach 1973). Similarly, with picorna viruses it seems that the former structure is present, as shown by electron microscopy of EMC virus RI (Thach et al 1974), or by carbethoxylating single-stranded regions of poliovirus RI before phenol extraction (Oberg and Philipson 1971, Ehrenfeld 1974). Studies with other RNA viruses have not been reported.

In conclusion, the finding that deproteinised RNA from RNA virus-infected cells contains dsRNA in RF and RI, and that the dsRNA sections of isolated RI are adequate to produce biological effects in vitro (f2 RI, Hunter et al 1972; Sindbis virus RI, Shenk and Stollar 1972; rhinovirus RI, Macnaughton et al 1976), does not mean that dsRNA exists in the cell, nor that it mediates effects in vivo. However, the ability to show some activities of dsRNA in non-deproteinised extracts of infected cells (Hunt and Ehrenfeld 1971) is strong evidence that dsRNA is produced in infection.

The situation with DNA viruses is rather different. Here there is no necessity for RNA of both senses to be transcribed, and replication complexes containing RNA-DNA hybrids do not have the same biological activities as dsRNA. However, there are some reports that small amounts of dsRNA are found in vaccinia virus infected cells (Colby and Duesberg 1969), although not in herpesvirus infected cells (Perez-Bercoff et al 1973). The vaccinia virus dsRNA is not only found in infected cell extracts, but is also found before deproteinisation, and in in vitro transcripts (Colby et al 1971); vaccinia cores can synthesise an inhibitor of protein synthesis, probably dsRNA, when incubated

in the reticulocyte lysate (H.R.B. Pelham, personal communication 1976). Thus it is not an artefact of phenol extraction.

dsRNA could arise by transcription of both strands of a given segment of dsDNA. Asymmetric transcription is known to occur in SV40, polyoma, and adenovirus infected cells (see Sambrook 1975), but the RNA sequences do not overlap in these cases, so pox viruses seem to be unusual in making self-complementary RNA. It is interesting that an endonuclease able to degrade dsRNA is induced as a late function in frog virus 3 infected cells, and is integrated into the virus (Kang and McAuslan 1972), so dsRNA may play a role in the replication of iridoviruses.

#### dsRNA as an inhibitor of *in vitro* translation

dsRNA is an efficient inhibitor of protein synthesis in reticulocyte lysates (reviewed by Hunt 1976) but is less active in other cell-free systems. Despite an initial report that *E.coli* cell-free translation was inhibited (Chao *et al* 1971), it now seems that prokaryotic systems are insensitive to (Jay *et al* 1974), and may even be stimulated (Bogaslowski *et al* 1976) by dsRNA. The wheat germ system (Reijnders *et al* 1975), ascites S-30 (Robertson and Mathews 1973), chick cell S-30 (Shenk and Stollar 1972), HeLa S-30 (Celma and Ehrenfeld 1974), and L cell S-30 (Kerr *et al* 1974b) are all relatively insensitive to dsRNA, concentrations of about 1  $\mu\text{g/ml}$  being required (Beuzard and London 1974) compared with 1 ng/ml in the reticulocyte lysate (Hunter *et al* 1972).

The relative insensitivity of these systems may be explained by the presence of nucleases able to degrade dsRNA (Robertson and Mathews 1973); by the presence of a factor able to overcome the inhibition (Balkow *et al* 1973; Kaempfer and Kaufman 1973); or by a range of other theories based on artefacts arising from preparation of the systems. On the other hand, L cell extracts can be made highly sensitive to dsRNA (Kerr *et al* 1974b), so different responses to dsRNA *in vitro* could reflect real differences in control systems operating *in vivo*. The high sensitivity of reticulocyte lysate to dsRNA

could be because the dsRNA control system is permanently switched "on", whereas in most other cells it is normally "off".

The mechanism of inhibition has been explored most deeply in reticulocyte lysates, and so far as is known, the same processes occur in the ascites S-30 (Robertson and Mathews 1973; Beuzard and London 1974). The chief characteristics of inhibition are: biphasic kinetics, with almost total shut off after a short lag (Hunter et al 1972); potentiation by preincubation (Hunter et al 1972; Robertson and Mathews 1973); and reversal by an initiation factor, IF-E2 (Balkow et al 1973, Kaempfer 1974; Beuzard and London 1974). In agreement with this, the lesion is at initiation of new polypeptide chains, with dsRNA preventing the binding of met tRNA<sub>F</sub> to 40S subunits (Darnbrough et al 1972), a process thought to be involved in normal initiation (Darnbrough et al 1973; Schreier and Staehelin 1973a, Sundkvist and Staehelin 1975). This binding is IF-E2 dependant (Schrier and Staehelin 1973a), so it was proposed that dsRNA was inactivating IF-E2.

Early work showed that a factor labelled IF-3 could bind strongly to dsRNA (Kaempfer and Kaufman 1973). That factor is now know to contain both IF-E2 and IF-E6 (equivalent to IF-MP and IF-M3, W. Merrick personal communication 1976) and both factors bind dsRNA although only IF-E2 overcomes the inhibition (Kaempfer 1974). An explanation of the inhibition based on inactivation of IF-E2 by dsRNA binding is thus unlikely, and the finding that bacterial IF-3 does not bind dsRNA (Jay et al 1974) is not an adequate explanation for the insensitivity of bacterial translation.

An alternative mechanism has been suggested (P. Farrell, K. Balkow, T. Hunt and R. Jackson, personal communication 1975) which embraces the inhibitory effects of dsRNA, hemin deficiency (Hunt et al 1972), and oxidised glutathione (Giloh and Mager 1975). Hemin control is mediated by an inhibitor (Gross 1974 a and b) which is formed in an ATP-dependant reaction (Balkow et al 1975) and inhibits formation of the met tRNA<sub>F</sub>-40S.GTP initiation complex (Legon et al 1973). Moreover, the inhibition is reversed by IF-E2

(Balkow et al 1973; Clemens et al 1974; Clemens et al 1975). A similar inhibitor was generated by dsRNA (Legon et al 1974), thus making direct interaction of dsRNA with the initiation factor unlikely.

The probable mode of action of these inhibitors has now been elucidated (P. Farrell, K. Balkow, T. Hunt, and R. Jackson, personal communication 1975; Ernst et al 1976). All treatments cause phosphorylation of a 35000 dalton polypeptide which is identical to the small subunit of IF-E2. This phosphorylation is prevented by a variety of purine derivatives known to reverse the inhibition of protein synthesis (Legon et al 1974). Moreover, phosphorylated IF-E2 is probably unable to bind met-tRNA<sub>F</sub> (B. Hardesty, personal communication 1976). The observation that formation, as well as action, of the inhibitor requires ATP (Balkow et al 1975) suggests that a series of protein kinases may be activated sequentially (Traugh et al 1976) to amplify the response.

Some of the more important features of this mechanism have been confirmed in Hela S-30, which is partly hemin dependant (C. Baglioni, personal communication 1976). Moreover, the finding that inhibition due to dsRNA proceeds by a similar mechanism to hemin and oxidised glutathione control argues for its physiological significance, for hemin control is important in intact reticulocytes (Hunter 1974; Hunter and Jackson 1975), and oxidising agents may be generated in energy-starved reticulocytes (Giloh and Mager 1975). However, a precise role for dsRNA in the reticulocyte is difficult to imagine, although some suggestions have been made (Downey et al 1976).

#### Inhibition of host cell protein synthesis by viruses

There are two problems here - firstly, what is the chemical nature of the protein synthesis inhibitor, and secondly, how does it discriminate against cellular protein synthesis whilst allowing viral protein synthesis to proceed?

The chemical nature of the inhibitor will be examined for the particular case of poliovirus infection, for here there is a variety of evidence on a possible role of viral dsRNA.

Willems and Penman (1966) investigated polyribosome run off in the presence or absence of an elongation inhibitor. The results implied either a lesion at, or before, polypeptide chain initiation, or degradation of mRNA. Gross degradation of mRNA can be excluded because extracted mRNA had the same sedimentation characteristics as mRNA from control cells (Willems and Penman 1966). There was no effect on the transit in time of ribosomes, or on the availability of charged met tRNA<sub>f</sub> (Summers and Maizel 1967). Detailed evidence is now available confirming that the lesion is at initiation, and is due to an inhibitor located in the ribosomal saltwash fraction of infected HeLa cells (Kaufmann et al 1976).

When Hunt and Ehrenfeld (1971) took cytoplasmic extracts of infected cells and assayed for inhibition of protein synthesis in the reticulocyte lysate system, they were able to isolate a macromolecular, heat-stable inhibitor, which appeared in parallel with inhibition of host protein synthesis in the infected cells (Ehrenfeld and Hunt 1971). Although resistant to trypsin, DNase, and ribonuclease, it was sensitive to alkaline digestion or micrococcal nuclease and showed other characteristics expected for the RF. dsRNA purified from infected cells had the same sedimentation constant and enzyme sensitivity as the inhibitor (Ehrenfeld and Hunt 1971). Purified virions can also inhibit the lysate, but only at high concentration (Racevskis et al 1976).

The presence of dsRNA able to inhibit cell-free translation in reticulocyte lysates does not mean that the RF or RI is responsible for host-cell shut off in vivo. Celma and Ehrenfeld (1974) attempted to quantitate the RF in infected HeLa cells in terms of its ability to inhibit protein synthesis in HeLa cell S-30s. They found that the effect of RF on in vitro synthesis was not sufficient to account for the inhibition of in vivo synthesis. However, because they used F-met tRNA<sub>f</sub> incorporation as a measure of initiation rate, they may have by-passed the inhibited step (Kerr et al 1974a,b, Content et al 1975), accounting for the apparently low inhibition.

Less direct evidence comes from experiments using mutants or metabolic inhibition. Cooper et al (1973) have proposed a model for the control of

poliovirus transcription and translation based on genetic studies. Basically, a precursor of the structural proteins acts as a regulator which binds to the 5' end of (+) sense RNA, preventing the completion of (-) sense strands, and allowing protein synthesis initiation on virus (+) RNA but not host mRNA. Processing of the precursor by cleavage to capsid proteins thus regulates (+) and (-) strand transcription, translation and maturation. Similar models, in which viral proteins regulate transcription, have been proposed for the (+) RNA coliphages (Weissmann 1974; Jay and Kaempfer 1975) and may explain the results with alphavirus ts mutants (H. Brzeski and S.I.T. Kennedy, personal communication 1976).

The finding that host-cell shut off maps in the structural protein genes (Steiner-Pryor and Cooper 1973) is thus not surprising. There was no correlation between the level of dsRNA produced and shut off, but there is always the problem that the level of detectability and the amount of dsRNA to inhibit protein synthesis may not be the same. Garwes et al (1975), in a survey of the same collection of ts mutants, concluded that the late cytopathic effect of poliovirus was due to RF formation, but it was unclear how protein synthesis inhibition was involved in this.

DI particles of poliovirus have a large deletion covering some of the structural gene precursor (Cole and Baltimore 1973). However, shut off of host synthesis occurs just as for the standard virus, suggesting that the structural gene product is not directly involved. Despite the deletion, viral RNA synthesis was normal, and could have caused the shut off.

The use of guanidine to inhibit viral RNA synthesis has also yielded confusing results. Guanidine prevents the bulk of viral RNA synthesis but shut off occurs normally (Bablanian et al 1965a,b). However, this does not exclude the RF as a candidate for protein synthesis inhibition because guanidine probably inhibits the release, not synthesis, of progeny RNA, and may thus increase the formation of RF (Baltimore 1967).

A final piece of evidence comes from the use of bovine enterovirus, which is related to poliovirus. BEV RF is not infectious, but is capable

of inhibiting host protein synthesis when added to uninfected cells (Cordell-Stewart and Taylor 1973a). It is not known whether exogenous dsRNA inhibits by the same mechanism as intracellular dsRNA, or whether the dose used reflected the quantity of RF normally formed during infection. These experiments show that dsRNA could mediate poliovirus-induced shut off, but cannot exclude other candidates.

Whatever the chemical nature of the inhibitor, the question of discrimination between host and viral synthesis remains. The finding that dsRNA inhibits in vitro translation of host and viral mRNAs equally (Robertson and Mathews 1973), is in keeping with a lesion prior to mRNA binding (Darnbrough et al 1972). How then, does dsRNA added to cells inhibit host, but not viral, mRNA translation (Cordell-Stewart and Taylor 1973a), and why does shut off by one virus not prevent translation of another virus (Kaluza et al 1976)?

It is important to realise that when different mRNAs are competing in the same system, their relative translation will be critically dependant on the overall initiation rate (Lodish 1974). Moreover, initiation seems to be normally rate-limiting in vivo (Lodish 1971, Lingrel and Woodland 1974), so the translation of mRNAs which have high-efficiency initiation sites is favoured. The relative translation of such mRNAs increases further when a non-specific block on initiation is applied (Lodish 1974). Thus, Lawrence and Thach (1974) have shown that in a competition situation in vitro, host-type (immunoglobulin) mRNA is less efficient than viral (EMC) mRNA. The same situation is found in infected cells, and the relative preference for viral rather than host mRNA is increased under conditions whence initiation is non-specifically inhibited by high salt (Nuss et al 1975). Viruses which produce highly efficient mRNA in large quantities could compete effectively without inhibiting total protein synthesis, but some viruses might benefit from producing a non-specific inhibitor of protein synthesis early in infection, when the quantity of virus mRNA is low (G. Koch, personal communication 1976).



This argument does not require mRNA-specific factors, just slightly different affinities of normal initiation factors for different mRNAs. It has been shown that the relative translation of different mRNAs competing in a fully-defined cell-free system can be varied at will by adjusting the conditions (McKeehan 1974), and those factors which had been identified as being mRNA specific (for globin mRNA, Lebleu et al 1972, Nudel et al 1973; or for EMC mRNA, Wible and Smith 1973) are now thought to represent non-specific initiation factors (Safer et al 1976), or factors required in different quantities by capped and uncapped mRNAs (M. Revel, personal communication 1976).

#### Viral cytopathic effects

It is possible that the cytopathic effects of some viruses are mediated by dsRNA. Cell killing is determined by an early virus function in Sindbis (Atkins and Lancashire 1976), VSV (Marques and Sakellick 1975), vaccinia (Bablanian 1968), polio (Bablanian et al 1975b), and reo (Subasinghe and Loh 1971) virus infection, as shown by the use of metabolic inhibitors and ts mutants. The virus functions required are still poorly characterised, but there is evidence that cytotoxicity is not simply a consequence of inhibition of host protein synthesis (Atkins 1976, Atkins and Lancashire 1976, Yamazaki and Wagner 1970, Collins and Roberts 1972, Garwes et al 1975). Some evidence concerning the cytopathic effects of picornaviruses will be given, to complement the preceding discussion on inhibition of host protein synthesis.

In a survey of ts mutants of poliovirus, Garwes et al (1975) found a strong correlation between late cytopathic effects and the synthesis of (-) RNA on the input (+) RNA. This suggested that formation of RF could be important. Unfortunately, DI particles of poliovirus, which produce normal quantities of dsRNA (Cole and Baltimore 1973), have not been characterised for cytotoxicity.

Other workers added protein synthesis inhibitors at various times during mengovirus infection and found that a "late" protein was responsible for cytotoxicity (Gauntt and Lockart 1968, Amako and Dales 1967). The differential effects of puromycin (which causes premature termination of

nascent polypeptides) and aminoacid analogues (which allow synthesis of non-functional proteins) suggested that cytotoxicity may be caused by a polyprotein precursor (Collins and Roberts 1972). Guanidine (which inhibits release of progeny RNA from the replication complex, Baltimore 1967) does not prevent the cytopathic effect of poliovirus when added 3.5 hr after infection, whereas puromycin does (Bablanian et al 1965b).

These results can be reconciled if there are two types of cytopathic effect. One, detected by trypan blue exclusion, is due to an early event in virus replication, possibly RF formation (Garves et al 1975). The other, detected in mengovirus infection by morphology changes or neutral red uptake (Amako and Dales 1967, Collins and Roberts 1972), or in poliovirus infection by morphology changes (Bablanian et al 1965b), may be due to synthesis of a "late" protein, possibly a polyprotein precursor. It is not unreasonable that different procedures for measuring cytopathic effects should yield different results - the effects seen by Garves et al (1975) did not involve lysosomal activation, whereas neutral red stain incorporation involves dye uptake by lysosomes (Allison 1967).

By adding RF to uninfected cells it was possible to show that dsRNA could cause cytopathic effects. Cordell-Stewart and Taylor (1971) showed that purified bovine enterovirus RF was toxic to cells, as well as inhibiting protein synthesis (Cordell-Stewart and Taylor 1973a). No infectious virus was produced, although the primary sequence of the RNA was important, as shown by the low toxicity of double-stranded homopolymers (Cordell-Stewart and Taylor 1971). Treating the RF preparation with nucleases to remove traces of ssRNA resulted in RF totally lacking in infectivity which could still cause cytopathic effects, even in the presence of protein synthesis inhibitors (Cordell-Stewart and Taylor 1973b). Thus, dsRNA can directly cause a cytotoxic effect, but as the cells absorbed about 100 times the amount of RF normally produced in infection, the cause of cytopathic effects during infection remains unclear. Whether this cytotoxicity resulted from protein synthesis inhibition (Cordell-Stewart and Taylor 1973a) or DNA

synthesis inhibition (observed by Teng et al 1973 in synchronised cells), and how it relates to the interferon-enhanced toxicity of dsRNA (Stewart et al 1972a), are all questions which need to be answered.

#### Summary

The evidence presented here suggests that dsRNA exists in low levels in normal cells, and in higher amounts in cells infected with some RNA and DNA viruses. It is able to inhibit translation in cell-free extracts of reticulocytes and interferon-treated cells, and less well in extracts of other mammalian cells. The mechanism for this inhibition is being investigated, and may be the same in different cell types. Non-specific inhibition of initiation could allow preferential synthesis of viral proteins, and dsRNA could be the molecule which causes virus inhibition of host synthesis in poliovirus infected cells. Involvement of dsRNA in virus cytopathic effects is less certain.

An involvement of dsRNA in the control of cell functions, and even in the control of virus infection in whole organisms, has been discussed before (Carter and de Clercq 1974, Metz 1975a), with similar conclusions. Other types of RNA, including the RNAs of endogenous oncornaviruses, small nuclear RNAs, and RNAs associated with mRNA, may also be important in the control of cell functions (Reaney 1975, Goldstein 1976, Reichman and Penman 1973, Heywood et al 1975, Bogdanovsky et al 1973).

### 1.7. AIM OF THE WORK

The preceeding sections have outlined the molecular basis for the actions of interferon and dsRNA, both in intact cells and in cell-free translation systems, and have related these effects to the possibility that virus-induced intracellular dsRNA is important in the control of macromolecular synthesis.

The observations that interferon treatment of L929 cells renders them susceptible to the toxic effects of dsRNA (Stewart et al 1972a) and to early cytopathic effects of vaccinia virus infection (Joklik and Merigan 1966, Stewart et al 1973a), as well as changing the protein synthesising machinery so that it is inhibited by dsRNA (Kerr et al 1974b), suggest that the control of protein synthesis is central to the effects of both vaccinia infection and dsRNA treatment. Thus when vaccinia virus infects an interferon-treated cell, the production of virus-specified dsRNA may trigger the activation of a protein synthesis inhibitor, which prevents the translation of viral mRNA. Inhibition of host-specified protein synthesis occurs by the normal mechanism, possibly mediated by dsRNA, and the cell rapidly dies as a result of early virus products or dsRNA. "Suicide" of the cell would preclude any virus production occurring through a leak in the interferon-induced block.

A similar mechanism could explain the toxicity of dsRNA for interferon-treated cells. Added dsRNA may be transported into the cell, there to trigger the formation of a translational inhibitor. Either as a result of this block, or because of the interaction of the dsRNA with some other structural or control system, the death of the cell follows.

This project set out to characterise the toxic effect in L929 cells and to find quantifiable biochemical changes occurring soon after dsRNA treatment, which might represent the initial events in the development of cytopathic effects. In particular, any changes which might occur in protein synthesis could be investigated both in vivo and in cell-free translation systems from parallel cultures. In vivo and in vitro changes could be correlated to test whether exogenous dsRNA could produce the same changes as dsRNA added to

cell-free extracts, and whether the in vitro effects gave any clues to the mechanism of the in vivo toxicity.

During the course of these experiments other lines of research were pursued. The chemical properties of the dsRNA used were explored, and the effects of various synthetic homopolymers were tested for inhibition of protein synthesis in the reticulocyte lysate. Because of the requirement for investigating in vivo and in vitro aspects in the same cells, a novel procedure for preparing cell-free systems from monolayer grown cells was devised, and the characteristics of the system investigated. Finally, the cytotoxic effect was described and the effects of dsRNA on in vitro translation characterised.

SECTION 2  
MATERIALS AND METHODS

## 2.1. MATERIALS

All inorganic chemicals were of analytical grade. The sources and specifications of some of the other materials used are listed here.

### Antibiotics and inhibitors

Edeine was from Calbiochem; actinomycin D from Merck, Sharpe and Dohme; D(+) threo chloramphenicol and cycloheximide from Sigma.

### Enzymes

Ribonucleases (Type IA) and T1, micrococcal nuclease, and pronase were from Sigma; deoxyribonuclease I (ribonuclease free) was from Worthington; creatine phosphokinase (Type I) was from Sigma.

Trypsin for cell passage and for biochemical experiments was Sigma Type III (from bovine pancreas). Soybean trypsin inhibitor (Type I-S, with an activity neutralising 1.4 mg trypsin/mg inhibitor) was also from Sigma.

### Chemicals

ATP, GTP, CTP, all aminoacids, E. coli tRNA (Type XXI), spermine tetrahydrochloride, sucrose (Grade I, for density gradients),  $\beta$ -mercaptoethanol, DTT, tris (Trizma base and Trizma HCl), Triton type N101, Hepes (free acid), hemin (Type I), bovine serum albumin, most of the proteins for molecular weight markers, DEPC, heparin, acetylphenylhydrazine, phenylhydrazine, EGTA (free acid), and 2-aminopurine were all from Sigma; most stains were from G.T. Gurr;  $K_2$ EDTA was from Koch-Light; Creatine phosphate from Boehringer; scintillation chemicals from Nuclear Enterprises; Soluene 350 from Packard; acrylamide and N,N'-methylenebis-acrylamide were from Kodak and were not normally recrystallised; ammonium sulphate was from Schwartz-Mann (ultrapure grade); ethanol from Franklin columns was Fisons "Spectrograde"

### Chromatography supports

Oligo dT cellulose was from Collaborative Research; CF-11 cellulose from Whatman; Sepharose and DEAE dextran from Pharmacia.

### Biological

Sera and media were from GIBCO Biocult or Flow Laboratories, some media were made in the laboratory. The anaesthetic used for the rabbits (2 Kg, male, NZW) was Intraval sodium.

### Polynucleotides

All homopolynucleotides were obtained from P-L Biochemicals and were made up in physiological salt. Specifications <sup>are</sup> in section 2.5.1. Reovirus dsRNA was the kind gift of L. Garcia.

P.chrysogenum dsRNA (BRL 5907) was the kind gift of C. Burbidge, Beecham Research Laboratories. EMC mRNA was the kind gift of A.E. Smith, ICRF.

### Radiochemicals

All radiochemicals were from Amersham. Some were diluted to lower specific activity before use.

L- [<sup>35</sup>S] methionine: normally about 200 Ci/mmole, 3-6mCi/ml

[<sup>32</sup>P] orthophosphate: carrier free, 10mCi/ml

adenosine 5' [ $\gamma$ -<sup>32</sup>P] triphosphate, ammonium salt: 10 Ci/mmole or greater

[5-<sup>3</sup>H] uridine 25-30 Ci/mmole, 1 mCi/ml

[methyl-<sup>3</sup>H] thymidine 5 Ci/mmole, 1 mCi/ml

L- [4,5-<sup>3</sup>H] leucine 40-60 Ci/mmole, 1 mCi/ml

<sup>45</sup>CaCl<sub>2</sub>: 0.9 Ci/mmole, 1.15 mCi/ml

<sup>86</sup>RbCl: 0.56 Ci/mmole, 3.86 mCi/ml

### Miscellaneous

The stock solution of Mg (OOCCH<sub>3</sub>)<sub>2</sub> used in most of the cell-free experiments was nominally 1.0 M and actually contained 1.05 M Mg(OOCCH<sub>3</sub>)<sub>2</sub> and 0.05 M K<sub>2</sub> EDTA.

The stock solution of hemin was made and checked according to Hunt and Jackson (1974).

The stock solution of 2-aminopurine is described in the legend to Fig. 6. Initiation factors were prepared according to section 2.3.10 or were the gift of T. Staehelin, Basel Institute for Immunology.



## 2.2. CELL CULTURE, VIRUSES AND INTERFERON

### 2.2.1. Media

Media were prepared according to the formulations given in the Flow Manual (Flow Laboratories Ltd. 1974), with the following modifications:

GMEM (Glasgow modification of Minimal Eagles Medium) was as specified with glutamine, non-essential aminoacids, and crystamycin added.

GMEM (Hepes) was GMEM without  $\text{NaHCO}_3$ , Hepes added to 20 mM and pH adjusted with NaOH.

GMEM-P contained no  $\text{NaH}_2\text{PO}_4$  (normal concentration in GMEM: 1.5 mM) and buffered with 20 mM Hepes

GMEM-M contained no methionine (normal concentration in GMEM: 100  $\mu\text{M}$ ) and buffered with 20 mM Hepes

GMEM/2% CS contained 2% newborn calf serum

GMEM/10% FBS contained 10% foetal bovine serum

PBS contained no  $\text{CaCl}_2$  or  $\text{Mg SO}_4$ .

trypsin/versene was 80  $\mu\text{g/ml}$  recrystallised trypsin, 80  $\mu\text{g/ml}$   $\text{Na}_2\text{EDTA}$  in PBS.

All media were sterilised by passing through a 0.22  $\mu\text{m}$  filter, and sera were mycoplasma and virus screened (Flow Laboratories Ltd).

### 2.2.2. Cell culture

NCTC 929 cells were obtained from GIBCO-Biocult at passage 566, 9 passages from American Type Culture Collection stock, and were passaged in 150  $\text{cm}^2$  bottles (Corning) every 5-8 days at 1 to 4 or 1 to 6 split. Monolayers were washed with PBS, trypsinised in about 10 ml trypsin/versene at 37°C for 5 min, and clumps dispersed by vigorous pipetting after the addition of 10 ml GMEM/10% FBS. Cells were normally counted using an improved Neubauer hemocytometer. Where required, the cells were spun out of the trypsin, resuspended in GMEM/10% FBS containing 10% DMSO and frozen at about 1°C/min, for storage in liquid nitrogen. Most of the experiments described were performed using cells 2 to 15 passages away from a frozen stock.

For experiments cells were seeded into plastic petri dishes, (Sterilin, tissue culture grade), or into thoroughly-washed glass scintillation vials arranged and sterilised in foil-covered metal racks. The numbers of cells seeded per culture so as to achieve confluence in 2 days were as follows:

140 mm petri dish;  $10 \times 10^6$  cells in 50 ml GMEM/10% FBS

50 mm petri dish;  $1 \times 10^6$  cells in 5 ml GMEM/10% FBS

25 mm glass vial;  $0.5 \times 10^6$  cells in 3 ml GMEM/10% FBS

10 mm glass vial;  $0.2 \times 10^6$  cells in 1 ml GMEM/10% FBS

Cultures were used shortly after reaching confluence at about  $0.3 \times 10^6$  cells/cm<sup>2</sup>.

The cells were screened periodically for mycoplasma contamination, using the fluorescent staining procedure (Russell et al 1975) or the uridine phosphorylase assay (Levine 1972).

#### 2.2.3. Viruses

NDV (strain F) was grown according to Moore and Burke (1974). About 10 HAU of seed virus (kindly provided by N. Moore) was injected into 11 day old embryonated eggs, and the allantoic fluid was harvested after 60 hr. The stock contained about 6000 HAU/ml.

SFV was grown up from a mouse brain stock (kindly provided by C.J. Bruton and S.I.T. Kennedy) in chick-embryo cell suspension as described (Kennedy and Burke 1972).

#### 2.2.4. Interferon preparation

The interferon stocks used for treating cultures were induced by NDV (strain F) in monolayer cultures of L929 cells, and were purified by the first step of the procedure described by Knight (1975).

About  $10^8$  L929 cells were treated with a mixture of 10 ml of GMEM/10% FBS and 10 ml of clarified allantoic fluid. Uninfected allantoic fluid was used for "mock" induction, or NDV (strain F) infected allantoic fluid was used for interferon induction. After 1 hr at 37°C for virus adsorption, the fluids were replaced with 50 ml of GMEM (containing no serum) and incubated overnight.

After 17-18 hr some cytopathic effects were evident in the infected cultures. The fluids were collected, clarified at 10000 g for 15 min, made to pH2 with HCl, and left 24 hr at 4°C. While still at 4°C and pH2, crystalline ammonium sulphate was added slowly to 24.1 g/100 ml, and the precipitate removed at 20000 g for 10 min. A further 15.1 g of ammonium sulphate was added to each 100 ml of supernatant, and the precipitate collected at 20000 g for 10 min. The pellet was dissolved in a small volume of PBS and dialysed against several changes of 200 volumes of PBS. The preparations were clarified at 40000 g for 15 min before diluting to 5 ml per  $10^8$  cells, and storing at -20°C in small aliquots.

The interferon preparations were titrated on several occasions using a laboratory internal standard and the NIH standard (G-002-904-511). Titres ranged from  $10^{5.3}$  to  $10^{5.9}$  u/ml (international units/ml) according to the preparation used and time of storage. The specific activity was about  $10^6$  u/mg protein, and mock preparations contained 50-100% as much protein as interferon preparations.

Occasionally, "crude" interferon was used. This was the laboratory internal standard, and was induced as described above but was not purified beyond pH2 treatment and clarification. It titred at  $10^{3.5}$  to  $10^{4.5}$  u/ml according to the preparation used.

#### 2.2.5. Interferon assay

Interferon was assayed in L929 cells using SFV challenge and the nucleic acid synthesis inhibition procedure described (Atkins et al 1974). Assays were normally performed using cell cultures grown in 25 mm scintillation vials, and the monolayers were solubilised, and scintillant added, to the vials. Some assays were performed using cell cultures grown in 10 mm vials, when the volume of interferon used was reduced to 0.5 ml, and volumes of virus, isotope, solubiliser, and scintillator were all reduced by half.

The NIH standard mouse interferon (G-002-904-511) routinely titred at  $10^{4.25}$  (18000)  $\text{INAS}_{50}\text{U/ml}$  compared with its defined titre of 12000 u/ml, so  $\text{INAS}_{50}\text{U}$  and u (international units) are equivalent to within a factor of 2. All titres are expressed in international units.

#### 2.2.6. Interferon induction by polynucleotides

Confluent monolayers in 50 mm petri dishes were treated for 16-18 hr with GMEM/2% CS containing interferon at  $10^{2.0} - 10^{2.5}$  u/ml, or an equivalent concentration of "mock interferon". After removing serum and interferon with three 2 ml washes of GMEM, 1.0 ml of GMEM containing 50 µg/ml of annealed poly rI.rC was added. After 50 min at 37°C this was removed, the cultures were washed once with 2 ml of GMEM, and fed with 2 ml of GMEM/2% CS. The fluids were harvested at 24 hr after induction or as required, and dialysed for 2 days at 4°C against 87 mM KCl, 13 mM HCl (pH2.0). The samples were neutralised by dialysis against PBS. Cell debris was removed at 2000 g for 5 min. If a 1x dilution was included in the assay, the final dialysis was against GMEM, and calf serum was added to the sample to 2% before making serial dilutions in GMEM/2% CS as usual.

The monolayers used for induction were normally fixed with 4% (v/v) formaldehyde in saline and stained with 1% gentian violet in 20% (v/v) ethanol for visual inspection of cytopathic effects.

#### 2.2.7. Estimation of cytopathic effect

Cultures were grown to confluence in 25 mm glass vials and treated for 16-18 hr with 1.0 ml of GMEM/2% CS containing  $10^{2.0} - 10^{2.5}$  u/ml of interferon or an equivalent concentration of "mock interferon". The cultures were then transferred to a circulating waterbath maintained at 37°C. After washing three times with 2 ml of warm GMEM, annealed poly rI.rC was added at 50 µg/ml in GMEM (Hepes). After 50 min the fluids were removed, the cultures washed with GMEM and fed with 2 ml of GMEM(Hepes). After a further 2.5 hr at 37°C the cultures were removed from the bath, washed three times with 2 ml PBS and fixed with 2 ml 10% TCA. The cultures were later washed with ethanol, dried, and solubilised in 0.50 ml 1M NaOH at 37°C for 1 hr. 50 µl was taken for protein determination.

Twelve cultures were used for each experiment, triplicate cultures being treated with both interferon and dsRNA (IFIC), interferon only (IF0), mock and dsRNA (MIC), and mock only (M0). The cytopathic effect was defined

as the average protein lost by the IFIC cultures:-

$$\text{i.e. } 100 \times \left( 1 - \frac{3 \times \text{IFIC}}{\text{IFO} + \text{MIC} + \text{MO}} \right)$$

#### 2.2.8. Radioactive labelling

25 mm vial cultures of cells were labelled with 0.5 ml of  $^{35}\text{S}$  methionine in GMEM-M,  $^{32}\text{P}$  orthophosphate in GMEM-P, and with  $^3\text{H}$  uridine,  $^{86}\text{RbCl}$ ,  $^{45}\text{CaCl}_2$ , or  $^3\text{H}$  thymidine in complete GMEM. The quantity of radioactivity used varied from experiment to experiment, but for routine estimation of acid soluble and insoluble incorporation 10  $\mu\text{Ci/ml}$   $^{35}\text{S}$  methionine or 10  $\mu\text{Ci/ml}$   $^3\text{H}$  uridine was used. When cell DNA was labelled to estimate the number of cells present,  $^3\text{H}$  thymidine was added at 5  $\mu\text{Ci/ml}$  during the 24 hr preceeding interferon treatment.

Pulses were normally stopped by removing the medium, transferring the vial to ice cold water, and washing rapidly three times with 2 ml of cold PBS. 1.0 ml of cold 10% TCA was added and acid soluble material extracted for at least 1 hr at  $4^\circ\text{C}$ . A sample of the TCA was taken for scintillation counting and the monolayer was washed a further two times with TCA, and then with ethanol, before drying and solubilising. If a sample was required for protein estimation, the monolayer was solubilised in 0.5 ml 1M NaOH and a sample counted in gel scintillator. Otherwise the monolayer was dissolved in 200  $\mu\text{l}$  of soluene 350 and counted in acidified toluene scintillator.

#### 2.2.9. Other procedures

Trypan blue excluding cells were counted in a hemocytometer after trypsinising 25 mm vial cultures of cells with 1.0 ml trypsin/versene, and adding 1.0 ml of GMEM/10% FBS and 0.5 ml 0.1% trypan blue in PBS.

Electronic cell counting made use of a Coulter counter (Coulter Electronics Ltd) set up with a 100  $\mu\text{m}$  orifice, amplification of  $\frac{1}{4}$ , current of  $\frac{1}{4}$ , and thresholds 10-100. This counted all material from  $0.3$  to  $3.7 \times 10^{-12}$  l in volume; the average cell volume was  $1.4$  to  $1.6 \times 10^{-12}$  l. The cells were prepared by trypsinising with 1.0 ml trypsin/versene, adding 1.0 ml GMEM/10% FBS,

breaking up clumps, and diluting to 20.0 ml with saline. The cells in a 500 $\mu$ l aliquot of the resulting suspension were counted.

Mitotic index was estimated from cultures which had been fixed in industrial methylated spirits, dried, and stained with Giemsa (freshly diluted 5 fold with H<sub>2</sub>O and centrifuged to remove particles). After 10 min staining the cultures were washed well and random fields of about 100 cells were photographed. The photographs were later used to estimate the number of cells in late telophase.

Photomicrography used on inverted microscope (Leitz Diavert) with phase ring, yellow filter, and 20 or 32x objectives. Panatomic X was rated at 50 ASA and developed in Acutol.

## 2.3. CELL-FREE SYSTEMS

### 2.3.1. Reticulocyte lysate

Rabbit reticulocyte lysate was prepared essentially according to Hunt and Jackson (1974).

2 Kg male rabbits were made anaemic by one of two methods. Either 5 daily injections with 0.5 ml of 2.5% v/v phenylhydrazine were followed by one day's rest and bleeding on day 7, or 4 daily injections with 2.5 ml of 1.25% w/v acetylphenylhydrazine was followed by bleeding on day 9. Bleeding was by cardiac puncture, yielding 100 ml of blood per rabbit. Samples were routinely sent to Mr. Raven, Pathology Laboratory, Warwick Hospital, for hematological analysis, and contained 90-100% reticulocytes with a hematocrit of 17-23%. There seemed to be no difference between the two injection schedules.

The heparinised blood was centrifuged at 2500 g for 15 min at 4°C. The red cells were washed three times in 130 mM NaCl, 5 mM KCl, 7.5 mM MgCl<sub>2</sub>, and the final pellet lysed with 1.5 packed-cell volumes of sterile distilled water. Debris was removed at 20000 g for 15 min at 4°C, and the supernatant dispersed and stored in liquid nitrogen.

### 2.3.2. Reticulocyte lysate high-speed supernatant

Reticulocyte lysate can be centrifuged so as to pellet most of the polyribosomes and globin mRNA, yet leave sufficient ribosomal subunits and initiation factors in the supernatant to give a mRNA dependant system (C. Preston, personal communication, 1975). This system was used in characterisation of mRNAs (section 2.4.4).

Lysate was made 25  $\mu$ M in hemin and centrifuged at 100000 g for 2 hr at 4°C. The supernatant was assayed as described below for total reticulocyte lysate, except that spermine was added to 80  $\mu$ M (Atkins *et al* 1975), and mRNA as required. This system showed about 3% of the endogenous activity of complete lysate, and was stimulated to about 32% of the activity of complete lysate by SFV 26S mRNA.

### 2.3.3. Micrococcal nuclease treated lysate

By using a  $\text{Ca}^{2+}$  dependant nuclease to degrade globin mRNA, the reticulocyte lysate can be made fully message-dependant (Pelham and Jackson 1976). This system was used for the assay of mRNA activity extracted from L929 cell-free systems, and was made as described (Pelham and Jackson 1976). It was assayed in the absence of polyamines.

### 2.3.4. Reticulocyte lysate assays

Total lysate, supernatant, or nuclease-treated lysate were all assayed by basically the same procedure. Hemin (see section 2.1) and creatine kinase (section 2.3.6) were added to the lysate to 25  $\mu\text{M}$  and 50  $\mu\text{g/ml}$  respectively. Preincubations with dsRNA were performed before adding the other components of the incubation.

Incubations were made up in 500  $\mu\text{l}$  plastic vials kept in ice-water. Additions were made to the lysate using a micropipette (Finpipette, Jencons Scientific Ltd) or microsyringe (Terumo, Shandon Southern Instruments Ltd, or Hamilton, Micromesure B.V) to give the following final concentrations of components. 10 mM creatine phosphate, 100 mM KCl, 0.5 mM Mg  $(\text{OCOCH}_3)_2$ , aminoacids and 70  $\mu\text{l}$  of lysate per 100  $\mu\text{l}$  incubation. Aminoacids were normally added at 50  $\mu\text{M}$ , with  $^{35}\text{S}$  methionine at 0.1 - 0.2  $\mu\text{M}$  and 30-60  $\mu\text{Ci/ml}$ . Alternatively, with  $^3\text{H}$  leucine present at 25 or 80  $\mu\text{M}$  and 16 or 50  $\mu\text{Ci/ml}$ , the remaining aminoacids were added according to their frequency in rabbit globin (Hunt and Jackson 1974).

Incubations were performed at 30°C in a pumped waterbath. When required, 2  $\mu\text{l}$  - 10  $\mu\text{l}$  samples were removed with a microsyringe and either diluted into 1 ml  $\text{H}_2\text{O}$  or deposited onto numbered squares of Whatman No. 1 filter paper.

Samples in  $\text{H}_2\text{O}$  were treated with 0.5 ml of 1M NaOH, 0.15M  $\text{H}_2\text{O}_2$ , 1 mg/ml leucine or methionine (according to isotope used) at 37°C for 15 min, before precipitating with 1 ml 25% TCA and filtering on GF/C filters. Filters were washed with a total of 30 ml per filter of 8% TCA and dried at 37°C before counting in toluene scintillator.



Samples deposited onto filter paper squares were allowed to dry for 30s before dropping into 10% TCA containing 1 mg/ml leucine or methionine. When all the samples were ready,  $H_2O_2$  was added to 0.015 M and the filters heated at 90-100°C for 20 min. The hot TCA was decanted and replaced with fresh 10% TCA 1 mg/ml leucine or methionine. After standing for 1 hr at room temperature the TCA was removed, and filters washed briefly with ethanol and ether before drying and counting.

For separation of labelled products by polyacrylamide gel electrophoresis, incubation mixtures were treated at 30°C for 15 min with 100 µg/ml RNase A and 10 mM  $Na_2EDTA$ , before making 1% in SDS and 2% in  $\beta$  mercaptoethanol and immersing in boiling water for 3 min. The precipitate of protein and potassium dodecylsulphate was redissolved by dialysis against a large excess of NURB (see section 2.6.2) before loading onto the gel.

#### 2.3.5. Detergent extracts from L929 cells

140 mm petri dish cultures of L929 cells were used when confluent. If required, the cells were treated with interferon or mock-interferon in 5 ml or 10 ml of GMEM/2% CS for 16-18 hr, and 5 ml of 50 µg/ml poly rI.rC in GMEM added after 3 × 10 ml washes with warm GMEM.

Lysis was performed as follows. Plates were removed individually from the incubator and immediately drained and rapidly cooled, by floating on ice-cold water and adding about 50 ml of ice-cold PBS. After a further two washes with PBS and one wash with cold KHMD (90 mM KCl, 30 mM Hepes KOH, 4 mM  $Mg(OOCCH_3)_2$ , 1 mM DTT, pH 7.4), the plates were drained for 30s and sucked dry. While the plate was floated horizontally on ice-cold water, five 100 µl aliquots of KHMD containing 0.1% v/v Triton N101 were added, distributed over the area of the plate. After 3 min, during which the detergent spread over most of the cells, the plate was tipped. Over the next 2 min the fluid was collected and transferred to a cold plastic vial (for liquid nitrogen storage, Sterilin). The extract was either assayed within 30 min or stored in liquid nitrogen.

### 2.3.6. Assay of detergent extracts

Incubations were performed in plastic vials (Sarstedt test tubes, 500  $\mu$ l capacity), using the following stock mixtures:

E containing 10 mM ATP, 1 mM GTP, 6 mM CTP, 100 mM creatine phosphate, 17 mM tris base, final pH 7.0-7.5.

-M containing nineteen aminoacids (no methionine) at 1 mM and 10 mM DTT.

CK containing 5 mg/ml creatine kinase in 50% v/v glycerol, 50% v/v 50 mM tris HCl pH 7.4.

Incubations were made up using 70  $\mu$ l extract, 10  $\mu$ l E, 5  $\mu$ l - M, 1  $\mu$ l CK, and  $^{35}$ S methionine, mRNA, and  $H_2O$  to a total of 100  $\mu$ l. Smaller volumes were used if required.

Final concentrations were thus:

63 mM KCl, 24 mM Hepes KOH, 2.8 mM  $Mg(OCOCH_3)_2$ , 0.07% v/v Triton N101, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 50  $\mu$ g/ml creatine kinase, 50  $\mu$ M each nineteen aminoacids, 1.2 mM DTT, and 2.2 mM tris, final pH about 7.4.

The ionic conditions are very approximate, being determined by the proportion of KHMD in the cell extract. The concentration of free  $Mg^{2+}$ , allowing for chelation by the nucleoside triphosphates, was about 1 mM. The total concentration of  $K^+$ , added as KCl and Hepes KOH, was about 75 mM.

Incubations were performed at 30°C in a circulating waterbath. Samples (2  $\mu$ l - 10  $\mu$ l) were taken and processed, as described in section 2.3.4 except that no  $H_2O_2$  was present.

### 2.3.7. Assay of protein phosphorylation in detergent extracts

Incubations contained, in every 100  $\mu$ l, 10  $\mu$ l of ATP  $^{32}P$  (20-90  $\mu$ Ci), 70  $\mu$ l of detergent extract, and 20  $\mu$ l of 50 mM KCl containing 0.5  $\mu$ g/ml dsRNA (BRL 5907) when required. The final concentration of added ATP was about 20-50  $\mu$ M. Incubation was at 30°C or 37°C for 15 min. Samples for acid precipitation were taken to 1 ml  $H_2O$  or to filter paper squares as described in section 2.3.4, except that the TCA contained 0.1 M  $Na_2P_2O_7$  and filtration

was on nitrocellulose filters (HAWP 45  $\mu$ , Millipore S.A., France) instead of GF/C, and no alkali was used.

Samples for polyacrylamide gel analysis were not ribonuclease treated but were made 1% in SDS and 2% in  $\beta$  mercaptoethanol and immersed in boiling water for 3 min. Free  $^{32}\text{P}$  orthophosphate was removed by exhaustive dialysis against NURB (section 2.6.2).

### 2.3.8. Preparation and assay of S-10 from suspension L929 cells

A cell-free extract was prepared from suspension grown L929 cells essentially as described by Clegg and Kennedy (1974). It was used, after preincubation and gel-filtration, for characterisation of mRNA (section 2.4.4). The assay system was as described (Clegg and Kennedy 1974) except that phosphoenolpyruvate was replaced by creatine phosphate and the pyruvate kinase by creatine kinase. KCl was added to 70 mM and  $\text{Mg}(\text{OCOCH}_3)_2$  to 4 mM total concentration.

### 2.3.9. Assay for inhibitor in detergent extracts

Inhibitors were formed in interferon-treated detergent extracts by preincubation with an energy supply and dsRNA, and were assayed in complete reticulocyte lysate containing 10  $\mu\text{g}/\text{ml}$  dsRNA.

Preincubations contained, per 100  $\mu\text{l}$ : 70  $\mu\text{l}$  of detergent extract or 0.1% v/v Triton N101 in KHMD, 1  $\mu\text{l}$  of CK, 10  $\mu\text{l}$  of E, and 20  $\mu\text{l}$  of 50 mM KCl containing 0.5  $\mu\text{g}/\text{ml}$  dsRNA (BRL 5907) as required.

After 30 min at 30°C, aliquots were removed to vials containing, per 100  $\mu\text{l}$ : 70  $\mu\text{l}$  of reticulocyte lysate (containing hemin and CK), 1  $\mu\text{l}$  of 1 mg/ml dsRNA (BRL 5907), 5  $\mu\text{l}$  of preincubation, and 24  $\mu\text{l}$  of a mixture of creatine phosphate, aminoacids, salts, and  $^{35}\text{S}$  methionine to give the final concentrations listed in section 2.3.4.

Incubation was at 30°C for 30 min. Samples were removed for acid precipitation as required.

### 2.3.10. Reticulocyte initiation factors

Rabbit reticulocyte polysomes were prepared from reticulocytes lysed as described by Schreier and Staehelin (1973b). Polysomes were pelleted from

the 12000 g supernatant at 100000g for 4 hr at 4°C. The pellets were resuspended and salt washed, and the supernatant from a 3½ hr, 160000 g spin collected. This was fractionated as described (Schreier and Staehelin 1973b) to yield fractions A and B1. The preparations were dialysed against Schreier and Staehelin's Buffer D containing 120 mM KCl and protein quantitated by extinction at 260 and 280 nm before storage in liquid nitrogen. Reticulocytes from 6 rabbits yielded 5 mg of fraction A and 20 mg of fraction B1.

The purified IF-E2 used by P. Farrell (section 7.5) was the kind gift of T. Staehelin.

## 2.4. mRNA PREPARATION AND CHARACTERISATION

mRNAs for cell-free translation were obtained from rabbit reticulocytes and SFV-infected BHK21 cells, and were characterised before use by translation in two different mRNA dependant cell-free systems.

### 2.4.1. Buffers

All solutions were treated with DEPC or autoclaved before use. Normal precautions against nucleases were taken.

TNE: 100 mM NaCl, 50 mM tris HCl, 1 mM Na<sub>2</sub>EDTA, pH 7.4.

TLES: 100 mM LiCl, 50 mM tris, HCl, 1 mM Na<sub>2</sub> EDTA, 0.1% SDS pH 7.4.

### 2.4.2. Globin mRNA

Red cells were prepared from one anaemic rabbit as described (section 2.3.1), and lysed with 2 packed-cell volumes of 4 mM Mg(OCOCH<sub>3</sub>)<sub>2</sub>, 6 mM  $\beta$  mercaptoethanol, 1.7  $\mu$ M DEPC (freshly-opened bottle). This was followed by hemin to 30  $\mu$ M and one volume of 1.5 M sucrose, 40 mM tris HCl pH 7.4. Debris was removed at 20000 g <sup>for</sup> 10 min at 4°C. Polyribosomes were pelleted at 100000 g for 4 hr at 4°C. The pellets were rinsed with 6 mM  $\beta$ -mercaptoethanol and resuspended in about 8 ml of ice cold 100 mM LiCl, 50 mM tris HCl, 1mM Na<sub>2</sub> EDTA, 2% SDS, 1% NDS, pH 7.4 using a loose-fitting dounce homogeniser.

The ribosomes were then extracted with 10 ml of TNE saturated phenol by shaking for 10 min at room temperature, and the phases separated at 3750 g for 10 min. The phenol layer was extracted with 10 ml TNE and the aqueous layers combined and extracted twice more with phenol and once with 95% v/v chloroform 5% v/v octan-1-ol.

Traces of phenol and chloroform were removed by three extractions with TNE-saturated ether, and residual ether blown off with dry nitrogen. The RNA was precipitated with 2.5 volumes of ethanol at -20°C, and pelleted at 3780 g for 20 min at 4°C.

PolyA(+) RNA was bound to oligo dT cellulose. A 2.5 ml bed-volume of oligo dT cellulose, which had been washed with 0.1 M NaOH and then with 10 mM tris HCl 0.1% SDS pH 7.4 at 37°C, was equilibrated with TLES at 0°C.

About 400  $OD_{260}$  units of RNA in 5 ml TLES were loaded and polyA(-) RNA washed off with TLES at 0°C, and then with TLES at 20°C. PolyA(+) RNA was eluted 37°C with 10 mM tris HCl 0.1% SDS pH 7.4. One quarter volume of 5 times concentrated TLES was added and the polyA(+) RNA purified by a further cycle through oligo dT cellulose.

The final yield of polyA(+) RNA was 4  $OD_{260}$  units. It was precipitated from the 10 mM tris HCl 0.1% SDS pH 7.4 by adding NaCl to 100 mM, ethanol to 70% v/v, and placing at -20°C. It was washed by pelleting, dissolving in  $H_2O$ , reprecipitating in the presence of 100 mM NaCl and 70% ethanol, and then washing with a mixture of 30% 100 mM KCl, 70% ethanol at -20°C.

Finally the RNA was dissolved in  $H_2O$  and adjusted to an extinction of 20 at 260 nm. It was dispensed and stored at -70°C.

#### 2.4.3. SFV intracellular RNA

Total polyA(+) RNA from SFV infected BHK21 cells (referred to as 'SFVmRNA') and a 26S fraction of this (referred to as '26S mRNA') were prepared from cells infected, harvested, and lysed as described by Clegg and Kennedy (1974). (Virus and cells were kindly provided by C.J. Bruton).

The cytoplasmic extract was deproteinised by three extractions with TNE-saturated phenol, one with 95% v/v chloroform 5% v/v octan-1-ol, and two extractions with TNE-saturated ether. Ether was blown off with dry nitrogen and the RNA precipitated in 70% ethanol at -20°C.

A portion of the RNA was purified by one cycle of oligo dT cellulose chromatography as described in section 2.4.2. The polyA(+) RNA ('SFV mRNA') was ethanol precipitated and washed as before, dissolved in  $H_2O$  to an optical density of 15 (260 nm) and stored in aliquots at -70°C.

The remainder of the RNA was loaded onto a 5-20% w/v sucrose gradient in TLES in the B XIV zonal rotor as described (Clegg and Kennedy 1974). The fractions containing 26S RNA were pooled and precipitated with 2.5 volumes of ethanol at -20°C. It was purified by one cycle of oligo dT cellulose

chromatography, precipitated, and washed as described (section 2.4.2). The 26S mRNA was dissolved in H<sub>2</sub>O to an optical density of 18 (260 nm) and stored in aliquots at -70°C.

#### 2.4.4. Characterisation of mRNA

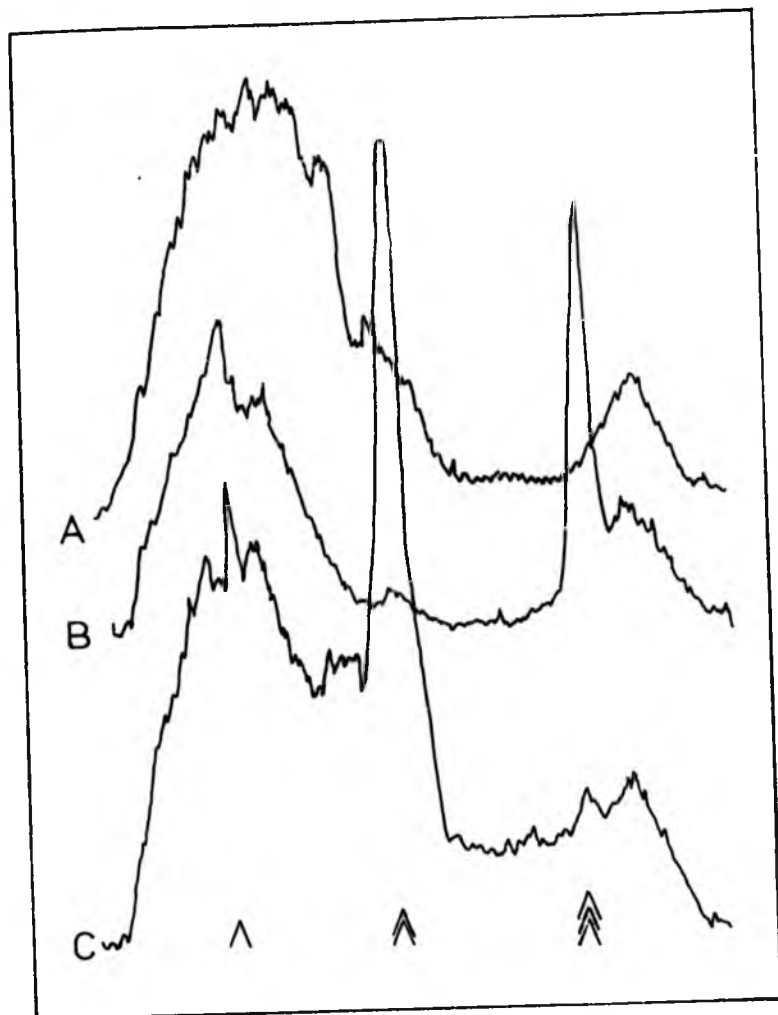
The mRNA preparations were characterised by translation in a preincubated L cell S-10 (Fig.1a) and a reticulocyte high speed supernatant (Fig.1b). Both sets of densitometer tracings show a high background at the top of the resolving gel, which appeared on the autoradiographs as a diffuse area of radioactivity and is probably due to free isotope. Apart from this, the 26S and SFV mRNAs coded for a product migrating with SFV capsid protein, and globin mRNA gave rise to a product migrating with rabbit globin. No other products were evident.

#### 2.4.5. Other RNA extractions

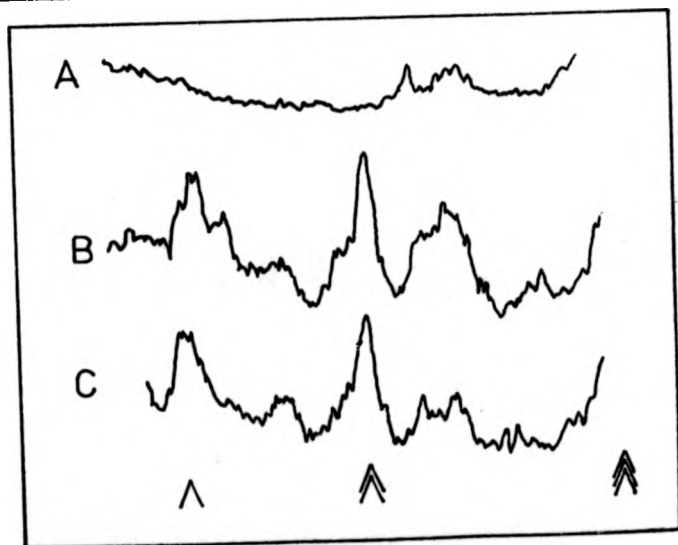
RNA was extracted from cell-free incubations (experiment described in Fig.37) using the method of Perry *et al* (1972).

50 µl aliquots of incubation mixture (section 2.3.6), containing detergent extract from <sup>3</sup>H uridine labelled cells and SFV mRNA, were taken to 1 ml of ice-cold TLES. 1 ml of a TNE-saturated mixture of 50% v/v phenol 50% v/v chloroform was added and shaken for 5 min at room temperature. The phases were separated at 3000 g for 10 min and the organic layer removed, leaving the interphase with the aqueous layer. After a further two extractions, the upper layer was extracted twice with TNE-saturated ether and precipitated with ethanol at -20°C in the presence of 50 µg *E.coli* tRNA. The precipitated RNA was dissolved in 100 mM KCl and reprecipitated before dissolving in 50 µl H<sub>2</sub>O and assaying for recovery of <sup>3</sup>H radioactivity and ability to stimulate protein synthesis. This extraction removed all but 0.01% of the <sup>35</sup>S radioactivity present in the initial incubations and allowed recovery of 60-80% of the <sup>3</sup>H radioactivity.

a



b





1. Characterisation of mRNA preparations.

Translation of globin mRNA (B) and 26S mRNA (C) in L cell 0, compared with endogenous (A). Incubations were performed (section 2.3.8) for 120 min before analysis on a 15% polyacrylamide gel (section 2.6.2). The gel was dried without fixation **or staining, and** autoradiographed for 36 hr. Approximate loadings: A, 33000 cpm; B, 32000 cpm; C, 44000 cpm.

Translation of 26S mRNA (B) and SFV mRNA (C) in reticulocyte 100, compared with endogenous (A). Incubations were performed (section 2.3.4) for 90 min before analysis on a 12.5% polyacrylamide gel. The gel was dried without fixation **or staining, and** autoradiographed for 72 hr. Approximate loadings: A, 26000 cpm; B, 45000 cpm; C, 48000 cpm.

The arrows show the top of the resolving gel (single arrow), and the positions of authentic SFV capsid protein (double arrow), and of authentic rabbit globin (triple arrow), run on parallel tracks.

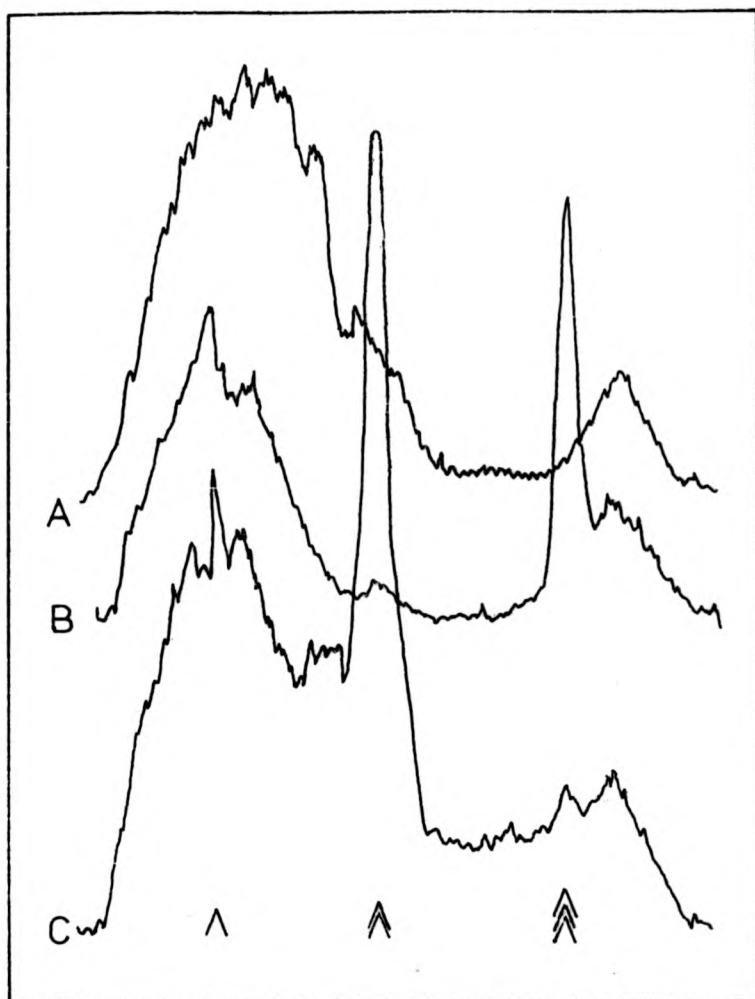
FIG. 1. Characterisation of mRNA preparations.

a) Translation of globin mRNA (B) and 26S mRNA (C) in L cell S-10, compared with endogenous (A). Incubations were performed (section 2.3.8) for 120 min before analysis on a 15% polyacrylamide gel (section 2.6.2). The gel was dried without fixation **or staining, and** autoradiographed for 36 hr. Approximate loadings: A, 33000 cpm; B, 32000 cpm; C, 44000 cpm.

b) Translation of 26S mRNA (B) and SFV mRNA (C) in reticulocyte S-100, compared with endogenous (A). Incubations were performed (section 2.3.4) for 90 min before analysis on a 12.5% polyacrylamide gel. The gel was dried without fixation **or staining, and** autoradiographed for 72 hr. Approximate loadings: A, 26000 cpm; B, 45000 cpm; C, 48000 cpm.

The arrows show the top of the resolving gel (single arrow), and the positions of authentic SFV capsid protein (double arrow), and of authentic rabbit globin (triple arrow), run on parallel tracks.

a



b

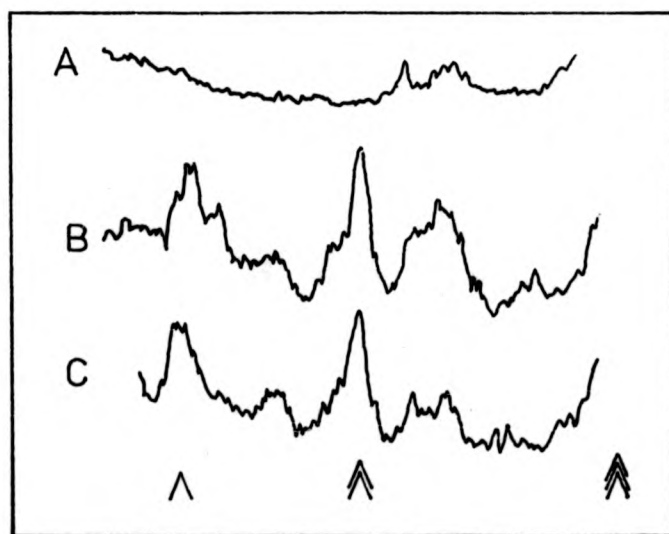


TABLE 1 : Degrees of polymerisation of "size kit" polynucleotides

Poly rI		Poly rC			
$S_{20,w}^a$	Degree of polymerisation <sup>b</sup> (nucleotides)	$S_{20,w}^a$	Degree of polymerisation <sup>b</sup> (nucleotides)		
c		c			
d		d			
1.0 < 2.5 < 4.5	27	47	1.5 < 3.08 < 4.7	51	75
2.1 < 4.39 < 8.2	96	136	1.9 < 3.88 < 5.9	80	115
3.2 < 6.13 < 9.4	203	259	3.1 < 5.94 < 8.2	204	260
3.4 < 7.94 < 16.0	350	416	4.9 < 8.20 < 11.8	404	478
5.3 < 10.6 < 27	642	730	7.0 < 10.5 < 15.0	674	767
6.2 < 12.5 < 24	913	990	8.0 < 13.2 < 18.1	1095	1180

- a)  $S_{20,w}$  given in manufacturer's specifications. limits are limits between which at least 80% of the polynucleotides sediment.
- b) degree of polymerisation calculated from molecular weight using nucleotide weights of 345 for rI and 321 for rC.
- c) calculated using formula  $M \approx 1.55 \times 10^3 \times S_{20,w}^{2.1}$  (Hu11 et al 1969)
- d) calculated using formula  $M = 2.82 \times 10^3 \times S_{20,w}^{1.9}$  (Tyte11 et al 1970)

## 2.5. POLYNUCLEOTIDES AND NUCLEASE DIGESTION

### 2.5.1. Specification of polynucleotides

Table 1 gives the manufacturer's specifications of the "size-kit" poly rI and poly rC, and the chain lengths calculated from sedimentation constants using two independent formulae (Hull *et al* 1969, Tyrell *et al* 1970). The two formulae disagree over the precise sizes of the low molecular weight polynucleotides, but the hybrids range from 30-50 up to 1000 base pairs. An indication of heterogeneity is provided by the limits between which more than 80% of the optical density sediments.

Other polynucleotides were specified as having molecular weights in excess of  $10^6$  daltons, apart from poly dI.dC which was greater than  $0.6 \times 10^5$  daltons. All contained negligible acid soluble material.

The molarities of polynucleotide solutions were calculated from published data (Chamberlin and Patterson 1965). The molarities given are the molarities of nucleotides, unless otherwise stated.

The molar extinction coefficient of poly rC at 300 nm was less than  $0.4 \times 10^3$ , confirming the absence of double-stranded structures (Chamberlin and Patterson 1965).

### 2.5.2. Annealing

Polynucleotides were dissolved in solutions containing 150 mM monovalent cations (either 150 mM NaCl, 50 mM tris HCl, 0.2 mM  $N_2$  EDTA pH 7.4, or PBS) and were annealed together at equimolar concentrations at 45°C for 1 hour. Duplexes were stored for short periods at 4°C, or for longer periods at -20°C. Frozen solutions were always annealed, either at 45°C for 30-60 min or briefly at 60-70°C, and left to cool before dilution or use. Solutions of poly rI also required heating before use.

### 2.5.3. Melting curves and spectra

Melting profiles and spectra of polynucleotides were obtained using a Unicam SP800 spectrophotometer fitted with a water-jacketted cuvette block.

Water temperature was maintained at temperatures up to 90°C by a waterbath (Haake, Berlin).

The temperature in a cuvette next to the sample cuvette was measured using an electronic thermometer (Comark Instruments Ltd) to within 0.5°C. The sample and reference solutions were contained in 3 ml or 1.5 ml silica cuvettes fitted with polypropylene stoppers, and at the end of each run the temperature was returned to 25°C to check that the concentration of polynucleotide had not been altered by evaporation.

#### 2.5.4. Nuclease digestion

Digestion of poly rI and poly rC by various concentrations of ribonucleases T1 and A were performed at 25°C in 50 mM tris HCl pH 7.4 solutions containing NaCl at concentrations from 150 mM to 800 mM. Samples were taken as required and precipitated with 0.5M HClO<sub>4</sub> in the presence of 500 µg BSA. After 1 hr at 4°C the acid insoluble material was removed at 4000 g at 4°C for 20 min and the acid soluble optical density read, relative to a blank containing no polynucleotides, in the Unicam SP600. The digestion products of poly rC were quantitated at 280 nm, and those of poly rI at 250 nm.

The standard conditions chosen for selective degradation of poly rI and poly rC but not poly rI.rC were 10<sup>-7</sup>g/ml RNase A, 300 u/ml RNase T1, 150 mM NaCl, 50 mM tris HCl pH 7.4 at 30°C for 1 hour, followed by acid precipitation as above. The digestion products of poly rI.rC were read at 263 nm, so that oligonucleotides from both strands should contribute equally to the acid soluble optical density.

Ribonuclease sensitivity was always expressed relative to complete digestion effected by either 1M KOH at 37°C for 2 hours, or by 50 µg/ml RNase A, 5000 u/ml RNase T1, 10 mM NaCl, 3 mM tris HCl pH 7.4 at 30°C for 1 hour.

## 2.6. FRACTIONATION PROCEDURES

### 2.6.1. Cellulose column chromatography of polynucleotides

Poly rI, poly rC and poly rI.rC were chromatographed on Whatman CF-11 cellulose in buffers containing ethanol according to the procedure of Franklin (1966).

Cellulose was defined and washed in 100 mM NaCl, 50 mM tris HCl, 10 mM Na<sub>2</sub>EDTA, 1%  $\beta$  mercaptoethanol pH 7.4 before equilibrating with 100 mM NaCl, 50 mM tris HCl, 1mM Na<sub>2</sub> EDTA pH 7.4 (TNE) containing 35% v/v ethanol (Spectrograde reagent, section 2.1). About 10 ml of cellulose was loaded into a 9 mm internal diameter column connected to a flow cell (UVicord II, LKB Produkter AB, Sweden) set to monitor the column output at 256 nm.

A sample containing about 10 OD<sub>260</sub> units of polynucleotide in 0.15 M salt was made 35% v/v with ethanol and loaded onto the column. The sample was washed in with three small aliquots of 35% v/v ethanol in TNE, and then with a large volume of the same buffer, until the optical density of the effluent fell to background. The column was then eluted with 15% v/v ethanol in TNE, and finally with TNE.

The fractions corresponding to each peak in the elution profile were pooled, the volume found, and the optical density at 260 nm measured using the relevant buffer as reference. The proportion of optical density in each fraction was thus calculated. Recovery of input optical density was (100  $\pm$  15)%.

### 2.6.2. SDS-polyacrylamide gels

Samples of cell-free incubations were prepared for gel electrophoresis as described in section 2.3.4, by exhaustive dialysis against NURB (40 mM tris base, 40 mM boric acid, 0.1% SDS, pH 8.6). Occasionally, the SDS treated incubations were made to pH 8 with NaOH and alkylated as described (Kennedy 1974) before dialysis against NURB.

Slab gels ( $200 \times 170 \times 1.8$  mm or  $160 \times 140 \times 1.8$  mm) were made using the discontinuous system described by Laemmli (1970). Basically, this uses a tris glycine (pH 8.4) electrophoresis buffer, a 4.5% polyacrylamide stacking gel containing 125 mM tris HCl at pH 6.8, and a resolving gel containing 275 mM tris HCl at pH 8.8.

The concentration of acrylamide was varied according to the separation required. The normal proportion of bis-acrylamide was 2.66% (w/w relative to acrylamide), which was reduced to 0.5% in high percentage gels to minimise cracking during drying down. Gradient gels were made using 20 ml of high concentration mixture (30% acrylamide containing 0.5% bis) in a constant volume mixing chamber, into which was pumped 60 ml of low concentration mixture (7.5% acrylamide containing 2.66% bis). The gradient was thus concave exponential, running from 7.5% at the top to 30% at the bottom.

A variety of different slot-formers were used, of which one with Teflon teeth was found to be best. Samples were loaded after the addition of sucrose to about 10% w/v and bromophenol blue to about 0.02% w/v. Electrophoresis was at 50V constant voltage or 14 mA, constant current, for 16-18 hours.

#### 2.6.3. Analysis of gels

Gels were stained, when required, by soaking for 2 hours in 0.05% w/v coomassie blue, 10% v/v acetic acid, 25% v/v methanol, and destained for the first 2 hours in 0.0025% w/v coomassie blue 10% v/v acetic acid 10% v/v methanol and then in 10% v/v acetic acid 10% v/v methanol, until fully destained. Alternatively, gels were fixed by overnight soaking in 10% v/v glacial acetic acid, 40% v/v methanol. Fixed or stained gels were washed in  $H_2O$  for 2-4 hours to removed volatile components before drying down onto Whatman 3 MM using a metal hot plate at about  $80^{\circ}C$  and a vacuum line.

Autoradiography was against Kodirex Medical X-Ray Film, developed in five times diluted Kodak DX80 and fixed in five times diluted Kodak FX40.



Fluorography of gels containing low amounts of  $^{35}\text{S}$  radioactivity was by the technique of Laskey and Mills (1975), in which the gel is impregnated with PPO before drying down and then exposed to 'pre-flashed' Kodak RPR40 film at  $-70^{\circ}\text{C}$ . 'Pre-flashing' was performed using a photographic enlarger rather than an electronic flash gun, to a background absorbance increment of 0.2.

Fluorography of gels containing low amounts of  $^{32}\text{P}$  radioactivity used a procedure suggested by R. Laskey (personal communication 1976). The gel was dried down without PPO treatment, and 'pre-flashed' Kodak RPR40 film was sandwiched between the gel and an intensifying screen (Kodak X-Omat, Regular) for exposure at  $-70^{\circ}\text{C}$ .

Autoradiographs and fluorographs were scanned using a Joyce-Loebl microdensitometer.

## 2.7. QUANTITATIVE METHODS

### 2.7.1. Protein estimation

Protein concentration was determined by the method of Lowry et al (1951). Samples containing 20-50  $\mu\text{g}$  of protein were diluted to 0.50 ml with water and mixed with 2.5 ml of a mixture of 100 parts 2% w/v  $\text{Na}_2\text{CO}_3$  in 0.1 M NaOH with 1 part 1% w/v sodium potassium tartarate and 1 part 2% w/v copper sulphate. After 10 min at room temperature 0.25 ml of a mixture of 1 part Folin and Ciocalteu's phenol reagent and 1.4 parts of water were added and immediately mixed. Optical density was read at 750 nm relative to a water blank. BSA in the relevant buffer was used as a standard.

Solutions containing Triton N101 formed a precipitate with the phenol reagent. This was avoided if the initial dilution to 0.50 ml used 0.1% SDS rather than  $\text{H}_2\text{O}$ . 0.1% SDS was also included in the BSA standards.

### 2.7.2. Liquid scintillation counting

Four scintillants were used according to the nature of the sample: Triton-toluene scintillator (0.4% w/v PPO, 0.005% POPOP, 33.3% v/v Triton X-100 in toluene) was used for aqueous samples.

Toluene scintillator (0.4% w/v PPO in toluene) was used for samples dried on glassfibre, nitrocellulose, or cellulose filters.

Acidified toluene scintillator (0.5% PPO, 0.01% POPOP, and 0.1% v/v glacial acetic acid in toluene) was used for sample which had been solubilised in soluene 350

Gel scintillator (0.7% PPO, 0.015% POPOP, 5% w/v naphthalene, 3.5% w/v silica gel, homogenised together in a mixture of 7 parts dioxan, 2 parts toluene, 0.3 parts ethanol) was used for samples which had been solubilised in alkali.

Samples were counted in a Packard Tri-Carb Scintillation Spectrometer. Double-labelling with  $^{35}\text{S}$  and  $^3\text{H}$  or  $^{32}\text{P}$  and  $^3\text{H}$  was used in some experiments.

Labelling conditions were chosen to give high  $^3\text{H}$  counts compared with  $^{35}\text{S}$  or  $^{32}\text{P}$  to minimise the effects of spillover. Samples labelled with one isotope only were included in each experiment for the calculation of spillover, and double-labelled samples were corrected assuming that this proportion was constant. When the efficiency of counting was required, a standard prepared in exactly the same way as the samples was included.

SECTION 3  
CHEMICAL PROPERTIES OF POLY rI.rC

### 3.1. CHEMICAL PROPERTIES OF POLY rI.rC: INTRODUCTION

The double-stranded complex between the homopolymers polyribinosinic acid (poly rI) and polyribocytidylic acid (poly rC) is a good interferon inducer (de Clercq 1974). Both homopolymers are readily synthesised using polynucleotide phosphorylase (Michelson *et al* 1967), and can be radioactively labelled. Being a synthetic polynucleotide it is not infectious, and chemical analogues can readily be made. It has thus become the dsRNA of choice for most interferon induction investigations and for studies on cell-surface binding of dsRNA. For these reasons, poly rI.rC was selected as a model dsRNA for use in the in vivo and in vitro experiments reported in the rest of this thesis.

There were two problems with the poly rI.rC preparations in use in the laboratory. Firstly, it was highly sensitive to nuclease degradation. Thus, when treated with 50 µg/ml RNaseA and 500 u/ml RNaseT1 in 0.15 M salt at 37°C for 30 min, conditions where SFV virion RNA is fully degraded but SFV RF is fully resistant, poly rI.rC was completely degraded. Secondly, radioiodinated poly rI.rC behaved anomalously on cellulose chromatography (M.D. Johnston, personal communication 1973). It was thus necessary to characterise the preparation to ensure it was double stranded, and not contaminated with unhybridised single strands of either poly rI or poly rC. These preparations were purchased as "double-stranded RNA".

### 3.2. RIBONUCLEASE DIGESTION

An assay for ribonuclease sensitivity (see section 2.5.4) was developed based on the measurement of acid soluble optical density. As a criterion for distinguishing poly rI.rC from free single strands, digestion conditions were found where poly rI.rC was fully resistant but poly rI and poly rC were fully degraded. This required the definition of the concentrations of two ribonucleases - RNaseA will degrade poly rC and RNase T1 will degrade poly rI - ionic strength, temperature, and time of digestion.

Initial experiments were performed following the kinetics of digestion at different RNase concentrations. The curves were expected to plateau at complete digestion. Lowering the nuclease concentration, or raising the salt concentration, should lower the rate, and any tendency to reach a plateau before complete digestion would imply the presence of two populations of RNA with different sensitivity.

Fig. 2 shows the digestion of poly rI.rC at different concentrations of RNaseA and T1, and two different ionic strengths. Under all conditions, poly rC was more sensitive than poly rI.rC, and there was no indication of contamination of the poly rI.rC with poly rC. Similar results were obtained when poly rI was compared to poly rI.rC (not shown).

Table 2 shows the extents of digestion of poly rI, poly rC and poly rI.rC using different ribonuclease concentrations but fixed ionic strength, time, and temperature. Using this data, the conditions finally chosen for selective ribonuclease digestion were 0.1  $\mu\text{g/ml}$  RNase A, 300  $\text{u/ml}$  RNaseT1, 150  $\text{mM}$  NaCl, 50  $\text{mM}$  tris HCl pH 7.4 at  $30^{\circ}\text{C}$  for 1 hr, followed by perchloric acid precipitation and measurement of acid soluble optical density at 263 nm. Under these conditions poly rI.rC was fully resistant, but poly rC and poly rI were fully degraded. This assay was used for the experiments in section 4.7.

FIG.2. Kinetics of ribonuclease digestion of poly rC and poly rI.rC.

Poly rC (a,b), or poly rI.rC (c,d), was mixed with various concentrations of RNase A and RNase T1 in 150mM (a,c) or 800mM (b,d) NaCl, 50mM tris HCl pH7.4. After various times at 25°C, aliquots were removed and precipitated (section 2.5.4). Acid soluble OD at 260 nm was expressed relative to complete digestion by alkali.

- ▲ control
- 0.1 µg/ml RNase A, 300 u/ml RNase T1
- 0.3 µg/ml RNase A, 1000 u/ml RNase T1
- 1.0 µg/ml RNase A, 3000 u/ml RNase T1
- 3.0 µg/ml RNase A, 10000u/ml RNase T1

acid soluble OD (%)

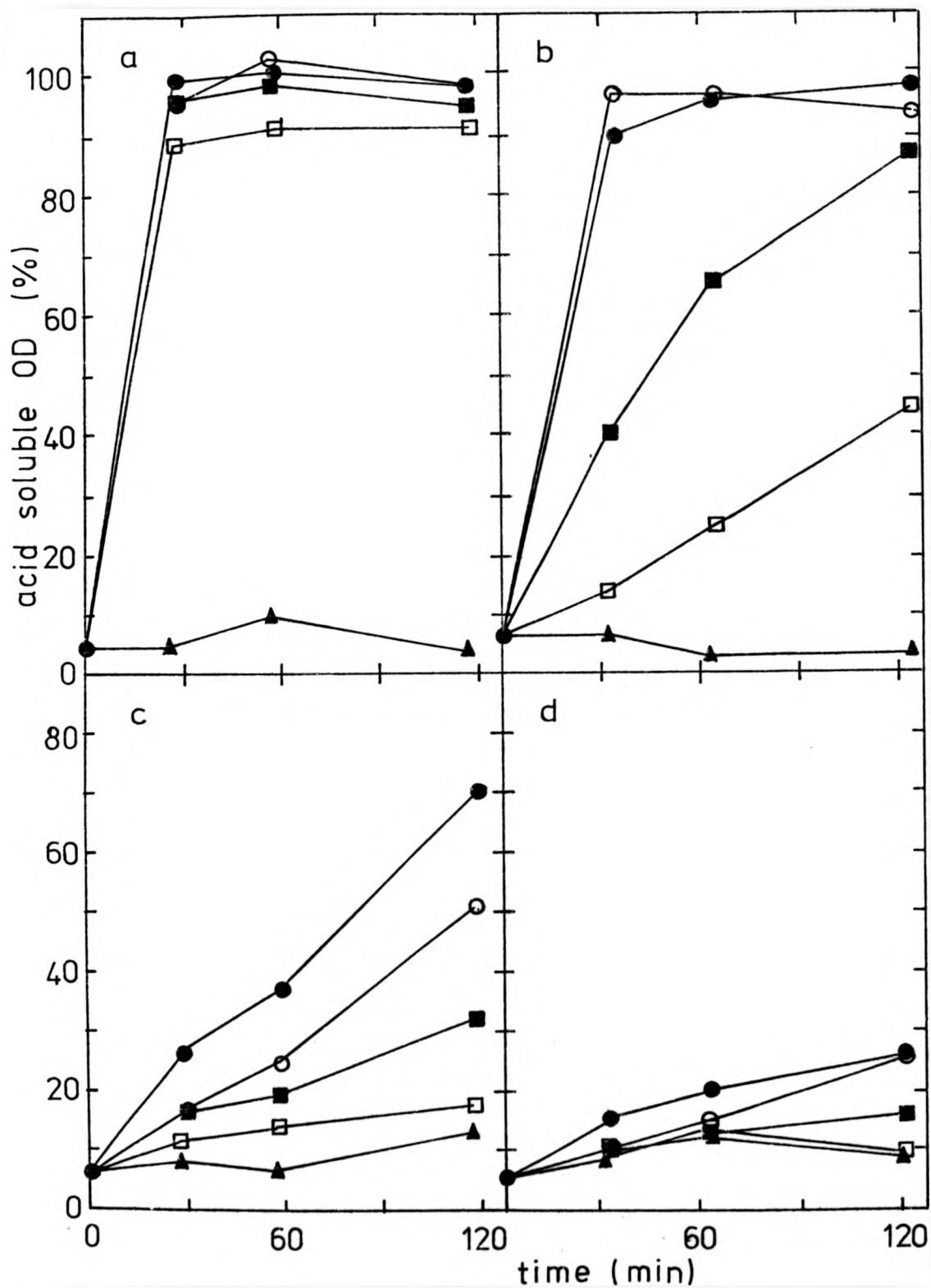




TABLE 2 : Comparison of sensitivities of poly rI, poly rC and poly rI.rC to different concentrations of RNase.

Concentration of RNase		% acid soluble OD		
A ( $\mu$ g/ml)	T1 (u/ml)	poly rI (250nm)	poly rC (280nm)	poly rI.rC (263nm)
3.0	10000	115	100	48
1.0	3000	112	100	28
0.3	1000	110	94	14
0.1	300	108	91	4

Digestion was carried out at 25°C in the presence of the indicated concentrations of RNase A and RNase T1 and 150 mM NaCl, 50 mM tris HCl pH 7.4, for 2 hr. Results are means of triplicates related to complete digestion by alkali. RNase T1 had no effect on poly rC and RNase A had no effect on poly rI.

### 3.3. CHROMATOGRAPHY OF POLY rI.rC ON CELLULOSE

dsRNA can be separated from DNA and ssRNA by chromatography on cellulose with buffers containing different proportions of ethanol (Franklin 1966). The sample is applied in buffer containing 35% v/v ethanol and all the RNAs, but not DNA or oligoribonucleotides, bind. ssRNA is eluted when the ethanol concentration is reduced to 15% v/v, and dsRNA is eluted in buffer containing no ethanol. Multistranded RNA may also elute in this fraction. (Macnaughton *et al* 1976).

Poly rI.rC chromatographs anomalously in this system. Fig.3a shows a comparison of poly rI.rC and reovirus dsRNA extracted from  $^3\text{H}$  uridine labelled virus. The poly rI.rC was annealed before mixing with the  $^3\text{H}$  reovirus dsRNA and applying to the column in 35% v/v ethanol. Although 82% of the recovered  $^3\text{H}$  eluted as dsRNA in the 0% ethanol fraction, the bulk of the optical density eluted as ssRNA (82% of recovered optical density in 15% v/v ethanol fraction). When the material eluted by 15% v/v ethanol was made 35% v/v in ethanol and rechromatographed, the profile was unchanged (Fig. 3b,c).

It was possible that the secondary structure of poly rI.rC was destroyed by 35% v/v ethanol. However, when the sample was applied in 15% v/v ethanol, only 2% of the optical density was eluted in the 0% ethanol fraction (results not shown). Moreover, when poly rI.rC in 15% v/v ethanol, and the 15% v/v ethanol fraction from the Franklin column, were compared with poly rI.rC in buffer alone, all showed the same melting profiles (with a melting temperature of 63.5 - 65.5°C and hyperchromicity of 68-76%) and the same spectra at 25°C and 72°C. All showed 100% resistance to nuclease digestion (conditions of section 3.2). When sedimented on sucrose gradients containing 150 mM NaCl and 50 mM tris HCl pH 7.4, but no ethanol, all sedimented at about 13S (data not shown).

These results show that poly rI.rC elutes from cellulose columns with the characteristics of ssRNA, yet this is not due to degradation or loss of secondary structure. This procedure clearly cannot be used to isolate poly rI.rC.

When phage f2 RNA is chromatographed at low temperature, the profile differs from that at room temperature (Engelhardt 1972), with a greater proportion running in the 0% ethanol fraction. It was possible that altering the temperature would alter the characteristics of poly rI.rC on the column.

When poly rI.rC was loaded onto a Franklin column at 4°C, 40-60% of the optical density routinely eluted in the 15% v/v ethanol fraction, and the remainder in 0% ethanol (Fig. 3d). No more material was eluted when the temperature was raised. Each of the fractions rechromatographed true (Fig. 3e,f). Under these conditions poly rC eluted in the 15% v/v ethanol fraction (Fig. 3g) but poly rI bound strongly even in 0% ethanol, and was only eluted at higher temperature (Fig. 3h).

Running the column at 4°C thus alters the elution profile of poly rI.rC. A greater proportion elutes as "dsRNA". About half of the material elutes as "ssRNA", and this would seem to be structurally different from the "dsRNA" because it chromatographs true. Both fractions could be contaminated with ssRNA, the 15% v/v ethanol fraction with poly rC, and the 0% ethanol fraction with poly rI.

The elution profile of poly rI.rC is thus dependant on both the proportion of ethanol and temperature. When poly rI.rC was adsorbed to the column in 35% v/v ethanol at 4°C, and the buffer changed to 15% v/v ethanol, about half of the material eluted (as in Fig. 3d). The temperature was then raised in steps of about 5°C, keeping the proportion of ethanol constant, and a second peak of absorbance eluted at 15-20°C. No further material eluted when the buffer was changed to 0% ethanol. Under these conditions poly rI eluted as a single peak at 35-40°C in the 15% v/v ethanol buffer.

FIG.3. Franklin column profiles

(a) Comparison of  $^3\text{H}$  reovirus dsRNA (broken line) and poly rI.rC (solid line) at  $20^\circ\text{C}$ . The column was loaded with  $8.5 \text{ OD}_{260}$  units of poly rI.rC and  $1700 \text{ cpm}$  ( $0.04 \text{ OD}_{260}$  units)  $^3\text{H}$  reovirus dsRNA.

(b,c) Re-chromatography of 15% V/V ethanol fraction.  $8.4 \text{ OD}_{260}$  units of poly rI.rC were chromatographed at  $20^\circ\text{C}$  (b). Peak B was taken, made 35% V/V in ethanol,  $2.0 \text{ OD}_{260}$  units loaded onto a second column, and chromatographed as before (c).

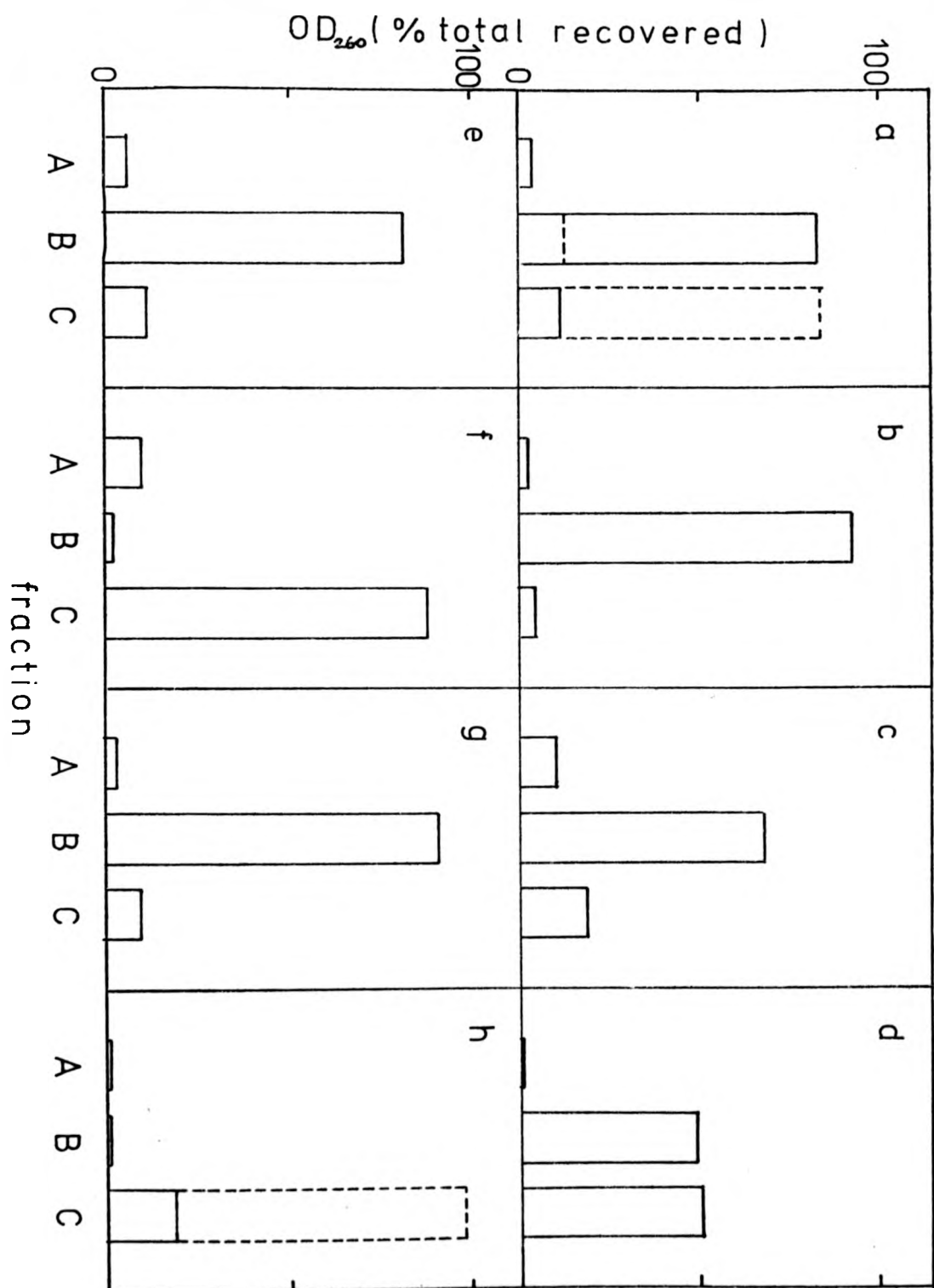
(d,e,f) Chromatography of poly rI.rC at  $4^\circ\text{C}$ .  $18.8 \text{ OD}_{260}$  units of poly rI.rC were chromatographed at  $4^\circ\text{C}$  (d).  $5.6 \text{ OD}_{260}$  units of peak B was made 35% V/V ethanol and rechromatographed (e).  $7.2 \text{ OD}_{260}$  units of peak C was made 35% V/V in ethanol and chromatographed on another column (f).

(g) Chromatography of poly rC at  $4^\circ\text{C}$ . The column was loaded with  $8.9 \text{ OD}_{260}$  units.

(h) Chromatography of poly rI at  $4^\circ\text{C}$ . The column was loaded with  $3.8 \text{ OD}_{260}$  units and chromatographed as before. At the end of the run, tightly bound optical density was eluted by raising temperature to  $25^\circ\text{C}$  (broken line).

In each case chromatography was by the procedure described in (section 2.6.1) and the total  $\text{OD}_{260}$  eluting in the 35% V/V ethanol (A), 15% V/V ethanol (B) and 0% ethanol (C) fractions is expressed as a percentage of the total recovered  $\text{OD}_{260}$ . In (a), samples were also taken for liquid scintillation counting.

fraction



### 3.4. DISCUSSION

Poly rI.rC is double-stranded as shown by its resistance to ribonuclease under conditions where poly rI and poly rC are fully degraded. It inhibits protein synthesis in the reticulocyte lysate (section 4.3) and induces interferon and causes a cytotoxic effect in interferon-treated cells (section 5). However, it is more sensitive to RNase A and RNase T1 than most viral dsRNAs and shows unusual elution characteristics on cellulose chromatography.

These findings might be common to all homopolymer duplexes or peculiar to poly rI.rC, and could result from base sequence or from size heterogeneity. For example, the high nuclease sensitivity could be due to partial endonuclease action with the nuclease following one strand of the duplex, or could be due to the fact that random cleavage of a short polynucleotide is more likely to produce acid soluble fragments than random cleavage of a long polynucleotide. The second explanation is unlikely with the size of polymer used here, although it may be important with short duplexes (section 4.7).

The reason for the unusual elution off cellulose columns is also unknown. At 4°C there seem to be two populations of poly rI.rC - one which elutes at 15% v/v ethanol, and one which binds more strongly and can either be eluted by raising the temperature or by lowering the ethanol concentration. Both populations elute together in the 15% v/v ethanol fraction when the experiment is performed at room temperature. The basis of this separation was not investigated, but may result if single-stranded tails of poly rI or poly rC were free to bind to the cellulose (De Larco and Guroff 1973).

The tight binding of poly rI to cellulose is not surprising. Poly rI is known to form multistranded complexes, particularly at low temperature (Thiele and Guschlbauer 1973; Zimmerman *et al* 1974). These complexes should be unstable under the ionic conditions used, but the presence of ethanol and polar groups on the cellulose could be important.

PolyrC only forms double-stranded structures at low pH and low ionic strength (Klump 1975). On the other hand, the tight binding of poly rI may represent a preference of the cellulose for polypurines (De Larco and Guroff 1973).

What is clear from these results is that the Franklin column cannot be used to purify poly rI.rC away from any free poly rI or poly rC. ssRNA contamination is high at both room temperature and in the cold. There are a number of alternative fractionation procedures available. Sepharose 4B has been used to separate dsRNA from ssRNA (Erikson 1969). However, no significant separation between poly rI.rC and poly rC or poly rI was achieved (data not shown). Methods which were not tried but may give good separation include precipitation with lithium chloride (Baltimore 1966) and fractionation on BD-cellulose (Stern and Friedman 1969).

SECTION 4  
THE EFFECTS OF POLYNUCLEOTIDES ON  
PROTEIN SYNTHESIS IN RETICULOCYTE  
LYSATES



#### 4. EFFECTS OF POLYNUCLEOTIDES ON PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES

##### 4.1. INTRODUCTION

It has been shown that dsRNA, but not ssRNA, inhibits protein synthesis in reticulocyte lysates (Ehrenfeld and Hunt 1971). dsRNA has other biological activities, including interferon induction (Tytell et al 1967) and toxicity for interferon-treated cells (Stewart et al 1972a), which show a requirement for stable secondary structure, high molecular weight, and 2' hydroxyl groups (de Clercq 1974).

Work on the inhibition of the reticulocyte lysate has been primarily from the point of view of mechanism, rather than the molecular requirements in the dsRNA structure, so it has involved a range of unrelated dsRNAs as inhibitors (for example poliovirus RF, Ehrenfeld and Hunt 1971; phage f2 RF Hunter et al 1972; and reovirion dsRNA, Darnbrough et al 1972). It was thus of interest to investigate the synthetic homopolymers commonly used in interferon research in terms of their ability to inhibit the reticulocyte lysate.

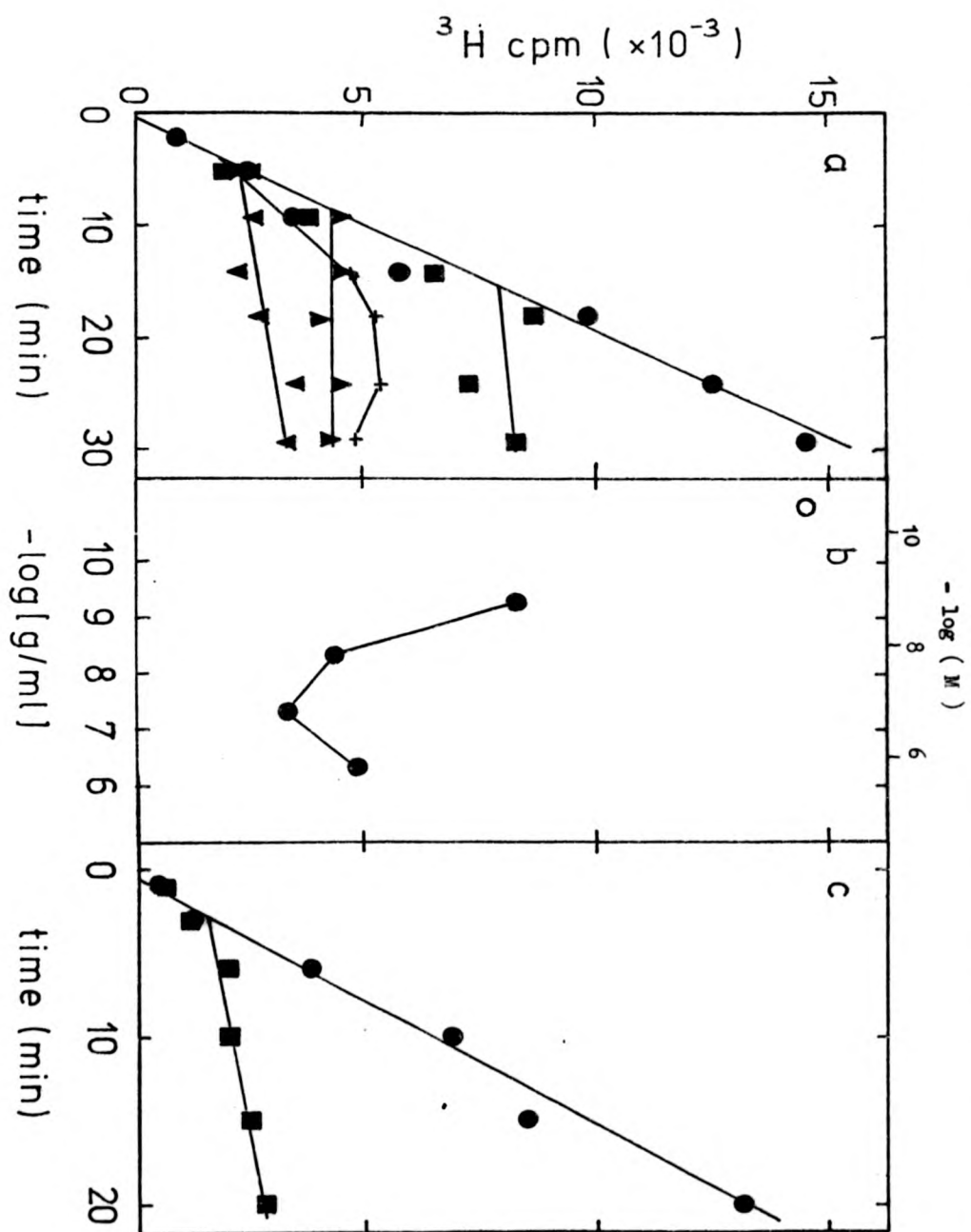
FIG.4. Effect of reovirus dsRNA and hemin on protein synthesis by the reticulocyte lysate.

Incubations were performed as described (section 2.3.4) with  $^3\text{H}$  leucine at  $80\ \mu\text{M}$  and  $16\ \mu\text{Ci/ml}$ , in the presence of various concentrations of reovirus dsRNA (a and b) or, using a different lysate, in the absence of hemin (c).  $5\ \mu\text{l}$  aliquots were taken at various times for acid precipitation and counting.

(a) Protein synthesis kinetics in the presence of  $25\ \mu\text{M}$  hemin and  $\circ$  0;  $\blacksquare$  0.5;  $\blacktriangle$  5;  $\blacktriangledown$  50; or  $+$  500 ng/ml reovirus dsRNA.

(b) The 30 min values from part (a) plotted against the concentration of dsRNA. The open circle is the control value.

(c) Protein synthesis kinetics in the absence of dsRNA in a lysate supplemented with  $\bullet$   $25\ \mu\text{M}$  or  $\blacksquare$  no hemin.



#### 4.2. CHARACTERISTICS OF THE LYSATE

The characteristics of inhibition of the lysate have been described (Hunter et al 1972). Like the inhibition resulting from the absence of hemin or the presence of oxidised glutathione, the inhibition due to dsRNA is characterised by biphasic kinetics, in which linear synthesis proceeds for a defined period, after which protein synthesis is almost totally shut off. The length of the lag period is reduced by increasing the concentration of dsRNA; but there is a limit above which the inhibition is reduced. The inhibition can be increased by preincubating the lysate with dsRNA before adding the other components required for protein synthesis (Hunter et al 1972).

The lysates used in this study were checked for these characteristics using various viral dsRNAs. Fig. 4 shows the kinetics (Fig.4a) and concentration dependence (Fig.4b) of protein synthesis in the presence of dsRNA extracted from reovirions. Maximal inhibition occurred at about 100 ng/ml, when the lag was about 5 min. For comparison, the effect of omitting hemin from the incubation is shown (Fig. 4c).

The effect of preincubation with dsRNA was studied (Fig.5), and the sensitivity of the assay was increased (Fig.5b) as previously reported (Hunter et al 1972).

Other virus RNAs have been assayed. For example, total RNA from Sindbis virus infected chick embryo cells inhibits with the characteristics of dsRNA, showing typical kinetics (Fig.6a), potentiation by preincubation (Fig.6b), and relief by 2-aminopurine (Fig.6a, Legon et al 1974). Total RNA from uninfected cells was not inhibitory at this concentration. We have also shown inhibition by rhinovirus RF and RI purified by Franklin column fractionation and lithium chloride precipitation from infected Hela cells (Macnaughton et al 1976).

FIG.5. Effect of preincubation on protein synthesis inhibition by reovirus dsRNA.

(a) 100  $\mu$ l incubations were made up containing reticulocyte lysate, 25  $\mu$ M hemin and reovirus dsRNA at various concentrations. At 0, 15, 40, or 60 min after the start of incubation at 30 $^{\circ}$ C, 20  $\mu$ l samples were removed to tubes containing 5  $\mu$ l of a mixture of all the components required for protein synthesis, to give a final concentration of 80  $\mu$ M and 16  $\mu$ Ci/ml  $^3$ H leucine. After a further 30 min at 30 $^{\circ}$ C, acid insoluble radioactivity was determined. The concentrations of dsRNA were:  $\bigcirc$  0;  $\square$  0.05;  $\blacktriangle$  0.5;  $\blacktriangledown$  1.0;  $\blacksquare$  5.0; and  $\bullet$  50 ng/ml.

(b) Using the same lysate as in part (a), 20  $\mu$ l preincubations were performed at 0 or 30 $^{\circ}$ C for 30 min before adding  $^3$ H leucine and other components and continuing incubation for 30 min. The results are expressed as a percentage of the incorporation in the absence of dsRNA, plotted against the concentration of dsRNA.

- $\bullet$  preincubation at 0 $^{\circ}$ C
- $\blacksquare$  preincubation at 30 $^{\circ}$ C.

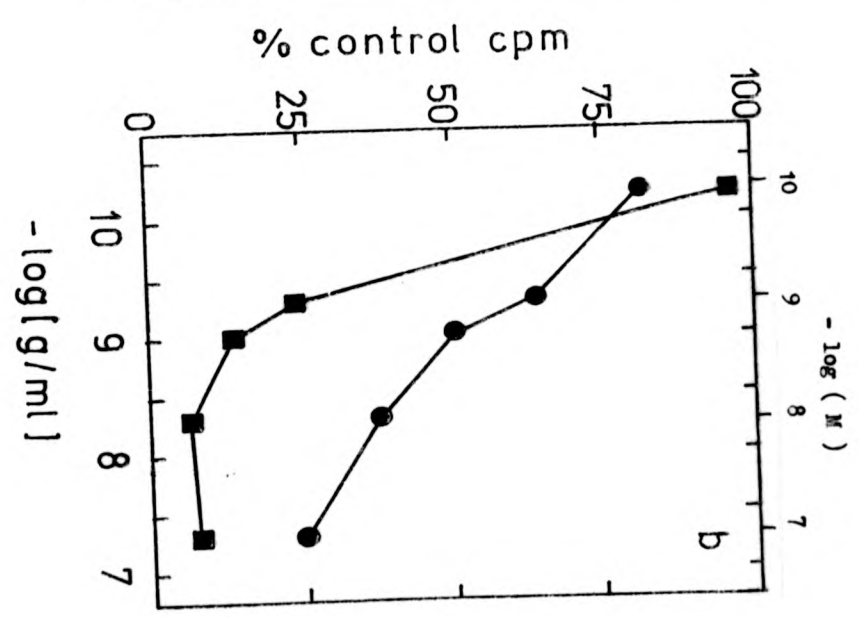
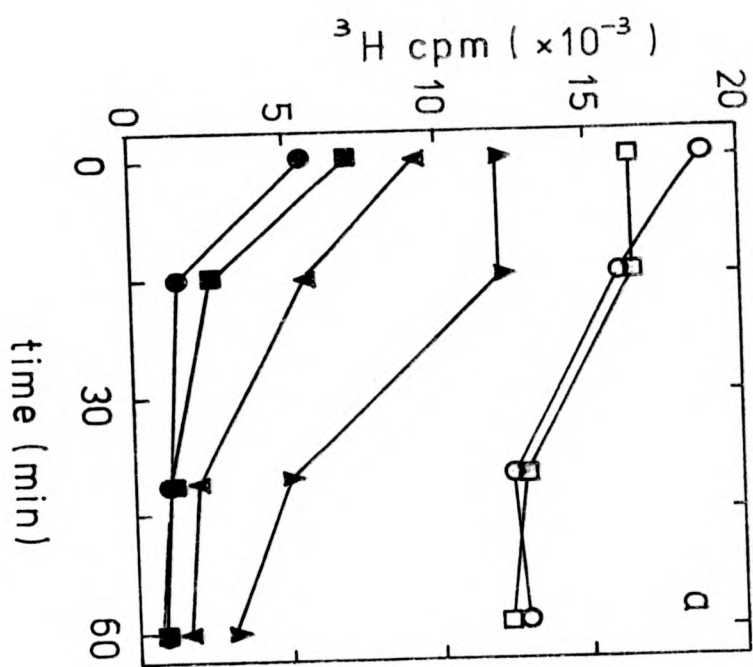


FIG.6. Inhibition of protein synthesis by RNA from Sindbis virus infected cells.

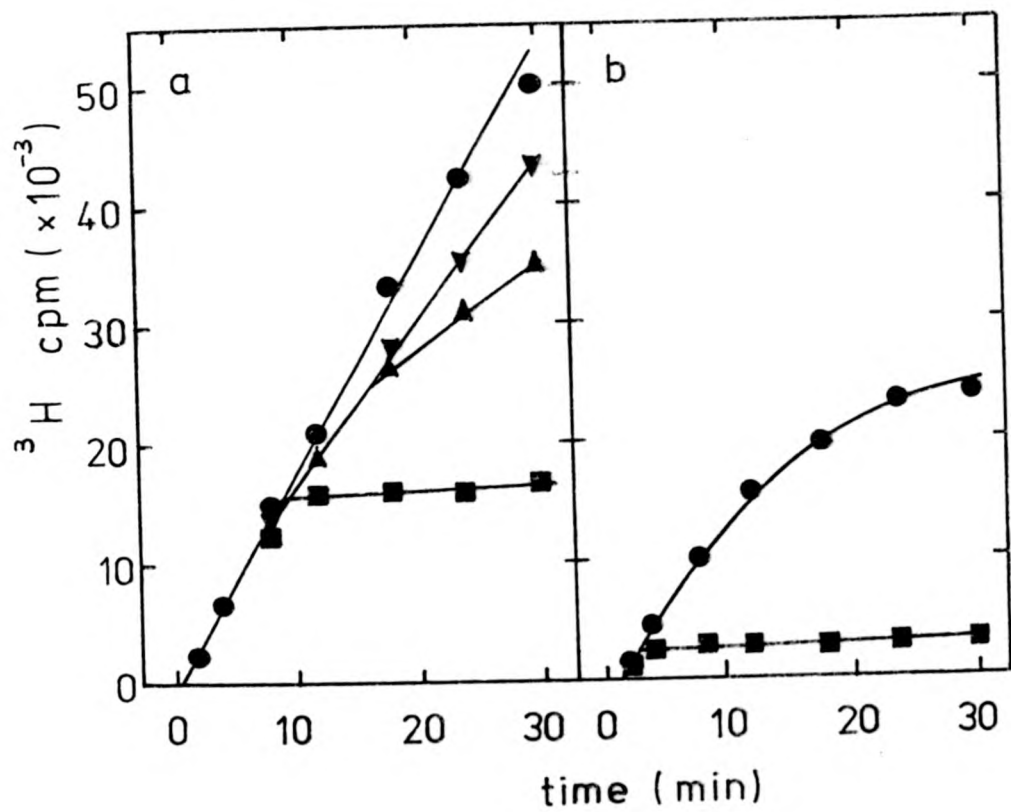
Chick embryo cells were infected with Sindbis virus and grown for 4 hr in the presence of 1  $\mu\text{g/ml}$  actinomycin D. The RNA was extracted, treated with DNase, and dissolved in 100 mM KCl, using 50  $\mu\text{l}$  for every  $10^6$  cells. This RNA was assayed for inhibition of the reticulocyte lysate at 10 or 100-fold dilution. RNA from mock-infected cells was not inhibitory unless preincubated with the lysate, when a 10-fold dilution inhibited 18%. The assays contained  $^3\text{H}$  leucine at 25  $\mu\text{M}$  and 50  $\mu\text{Ci/ml}$ , and 3  $\mu\text{l}$  aliquots were taken for acid precipitation.

(a) RNA from infected cells assayed without preincubation in the presence or absence of 2-aminopurine. 2-aminopurine was dissolved in water to about 140 mM and neutralised with KOH (final  $\text{OD}_{306} = 810$ ). It was diluted 20-fold in the assay, to about 7 mM.

- control
- + 10-fold diluted RNA
- ▼ + 2-aminopurine
- ▲ + RNA + 2-aminopurine

(b) RNA from infected cells diluted 100-fold into the lysate was preincubated at  $30^\circ\text{C}$  for 30 min before the addition of the other components for protein synthesis.

- control
- + 100-fold diluted RNA.





#### 4.3. INHIBITION BY POLY rI.rC

Poly rI.rC has been reported to inhibit the reticulocyte lysate (Ehrenfeld and Hunt 1971). However, with a number of lysates I found the inhibition at low concentrations to be irregular and difficult to reproduce. At concentrations in the range of  $10^{-8}$  to  $10^{-4}$  M ( $3 \cdot 10^{-9}$  to  $3 \cdot 10^{-5}$  g/ml) - inhibition after 30 min incubation was never more than 30%. "Activating" the dsRNA, by incubating in a buffer containing magnesium ions (de Clercq et al 1971; de Clercq and Merigan 1971), before adding to the lysate did not increase the inhibition.

However, when the poly rI.rC was preincubated with the lysate, inhibitions of up to 95% were observed, representing inhibition within 2 min of the addition of components for protein synthesis (Fig.7). 80% inhibition was observed with only 1 ng/ml dsRNA (compare Fig.5b and 6c).

Poly rI.rC thus behaves like a classical dsRNA inhibitor in preincubated systems, but no conclusions could be drawn about non-preincubated systems. The following experiments were performed after preincubation, unless otherwise stated.

FIG.7. Inhibition of protein synthesis by poly rI.rC

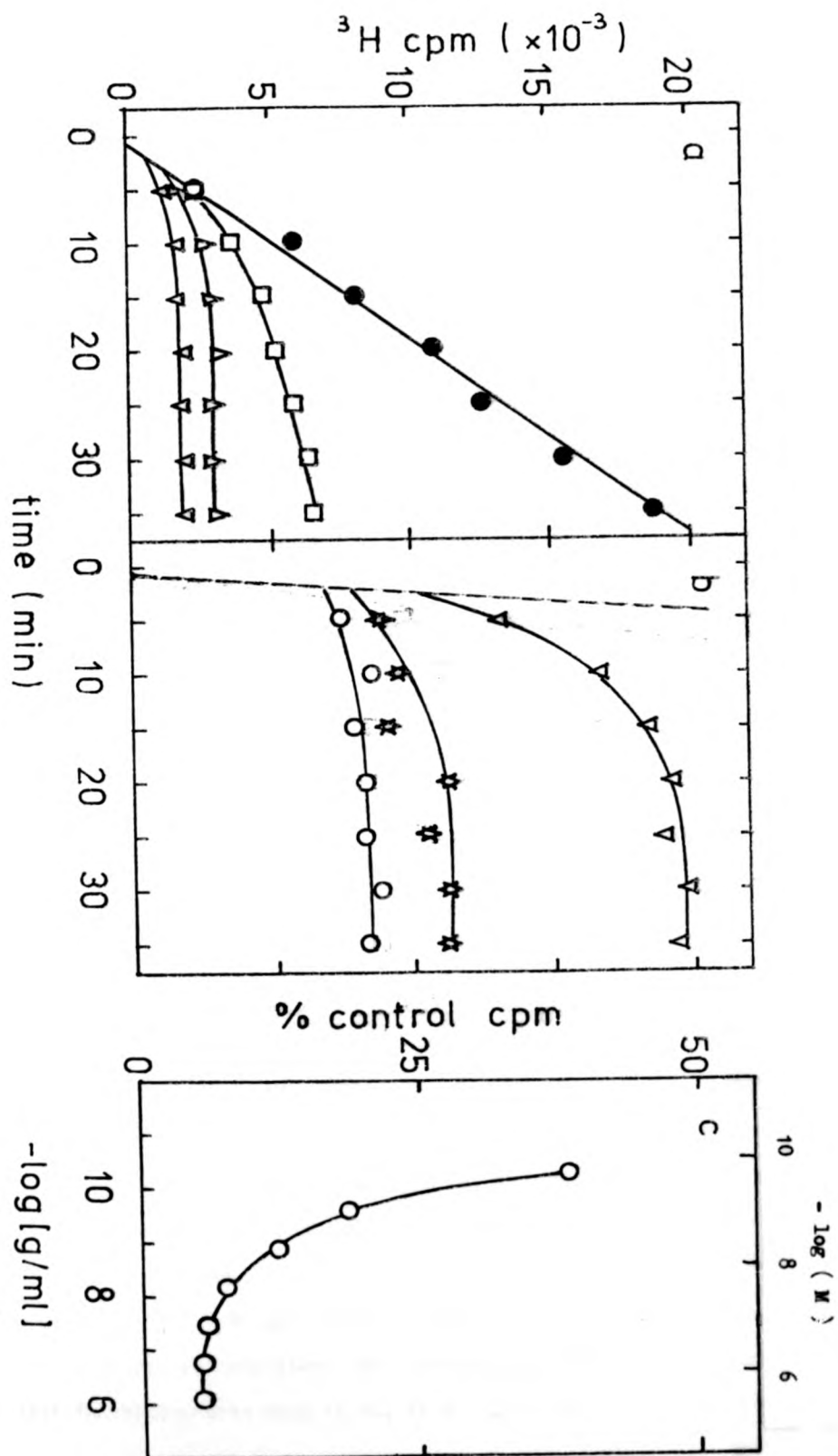
Poly rI.rC was diluted as required and added to the reticulocyte lysate. After 30 min at 30°C the other components for protein synthesis were added, including  $^3\text{H}$  leucine at 80  $\mu\text{M}$  and 50  $\mu\text{Ci/ml}$ , and incubation continued. 5  $\mu\text{l}$  aliquots were taken as required and acid insoluble radioactivity determined.

(a,b) Kinetics of protein synthesis. The ordinate scale has been expanded 10-fold in part (b) to show the inhibition caused by high concentrations of poly rI.rC, and the control line from part (a) is shown.

- ⊙ control
- 0.2 nM poly rI.rC
- △ 1.0 nM
- ▽ 5.0 nM
- ☆ 27 nM
- 135 nM

Results using 675 nM or 3380 nM could not be distinguished from those shown for 135 nM.

(c) The 30 min time points from parts (a) and (b) plotted as percentage of control incorporation against concentration of poly rI.rC.



#### 4.4. COMPARISON OF DIFFERENT HOMOPOLYMERS

To verify a requirement for a double-stranded structure with 2' hydroxyl groups on both strands, a comparison of poly rI.rC, poly dI.dC, poly rI.dC, poly rI and poly rC was made. Poly dI.rC has a melting temperature in 100 mM NaCl of only 35°C (Chamberlin and Patterson 1965) and would only be marginally stable in the lysate. Although poly rI and poly rC can each form multistranded complexes under certain conditions (Thiele and Guschlbauer 1973, Klump 1975) this should not occur in the lysate.

Fig. 8 shows that dsRNA is more inhibitory than dsDNA, DNA.RNA, or ssRNAs, and is the only polynucleotide showing biphasic kinetics.

In view of the failure of tsRNA to induce interferon in primary rabbit kidney cells, even though it competes with dsRNA for the interferon-inducing receptor (de Clercq et al 1974), it was of interest to see whether tsRNA could inhibit the lysate. Triple-stranded complexes of poly rI with poly rC are not stable under the conditions of interferon induction or in the lysate (Thiele and Guschlbauer 1969), so a non-homologous tsRNA was used.

Poly rA.rU.rU has been characterised (Michelson et al 1967) and no disproportionation to dsRNA occurs in 150 mM NaCl at 37°C (de Clercq et al 1975b). When assayed at 11 µM (in the same experiment as Fig.8) poly rA.rU.rU inhibited 54%, compared with 81% for 1 µM poly rI.rC. The inhibition was comparable with that due to poly rI. However, when assayed in another lysate after 60 min preincubation, it was found that 11 µM poly rA.rU.rU could inhibit with biphasic kinetics (Fig. 9a,b). This effect depended on preincubation (Fig.9c). Knowing the dose-response curve for poly rI.rC, and assuming it is the same for poly rA.rU, the inhibition can be explained by contamination of the 11 µM poly rA.rU.rU with about 0.1 µM poly rA.rU.

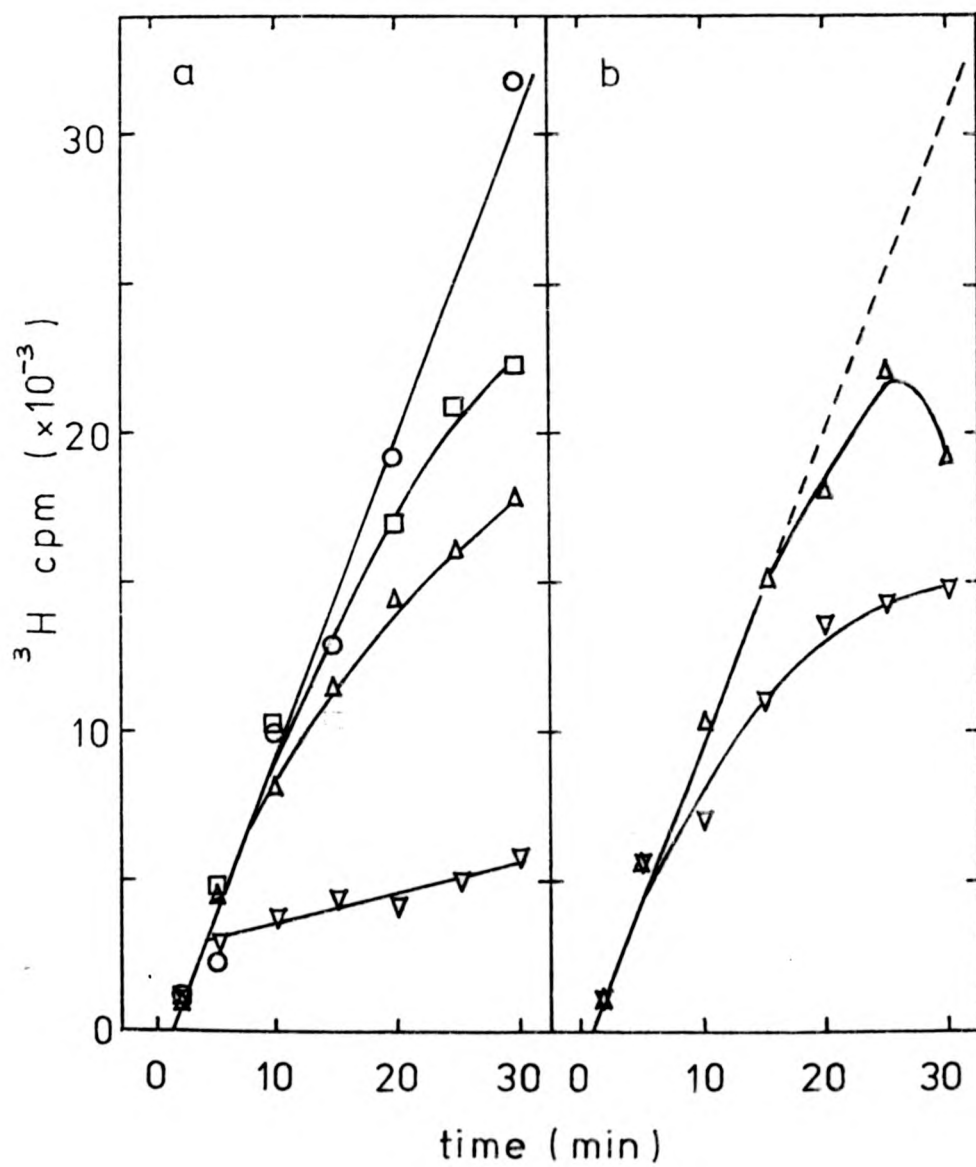
It has been reported (de Clercq et al 1974) that tsRNA can prevent induction by dsRNA. As poly rA.rU.rU and poly rI.rC do not exchange strands under physiological conditions (de Clercq et al 1975b), and poly rA.rU.rU is less inhibitory than poly rI.rC, it was possible to perform an analogous

FIG.8. Inhibition of protein synthesis by single and double-stranded homopolynucleotides.

Polynucleotides were added to the lysate to a final concentration of 1  $\mu$ M (in nucleotides). After 30 min at 30°C components for protein synthesis were added, including  $^3$ H leucine at 25  $\mu$ M and 50  $\mu$ Ci/ml. 5  $\mu$ l aliquots were taken as required for acid precipitation. Parts (a) and (b) represent data from the same experiment.

(a) ○ control; □ + poly rI.dC; ▲ + poly dI.dC; ▼ + poly rI.rC

(b) ▲ + poly rC; ▼ + poly rI.



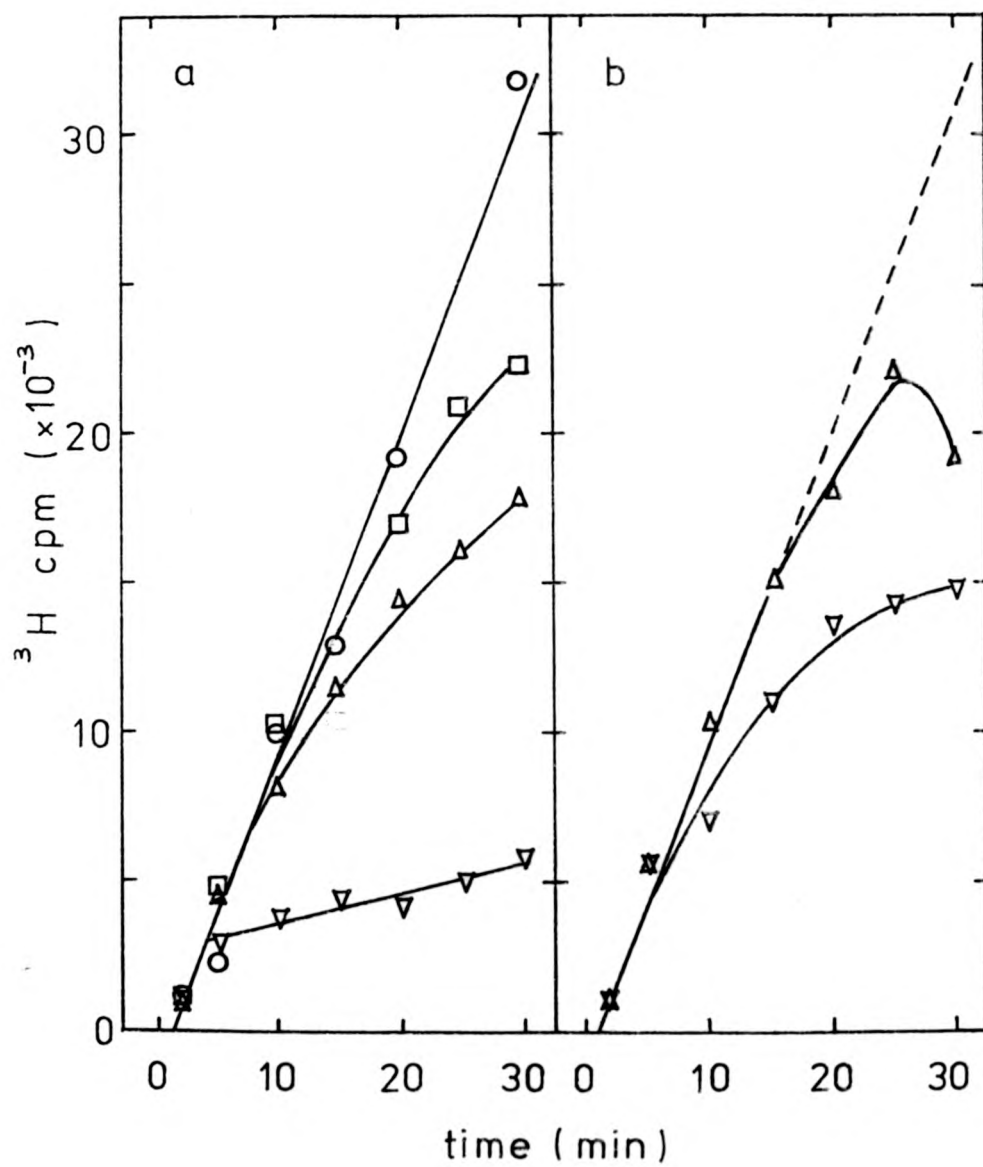


FIG.9. Inhibition of protein synthesis by poly rA.rU.rU

Polynucleotides were added to the reticulocyte lysate at 1  $\mu$ M (poly rI.rC, poly rI, or poly rC) or at 11  $\mu$ M (poly rA.rU.rU) final concentration. After various times of preincubation at 30°C, aliquots were removed and other components for protein synthesis were added, including  $^3$ H leucine to 25  $\mu$ M and 50  $\mu$ Ci/ml. 5  $\mu$ l aliquots were taken as required for acid precipitation.

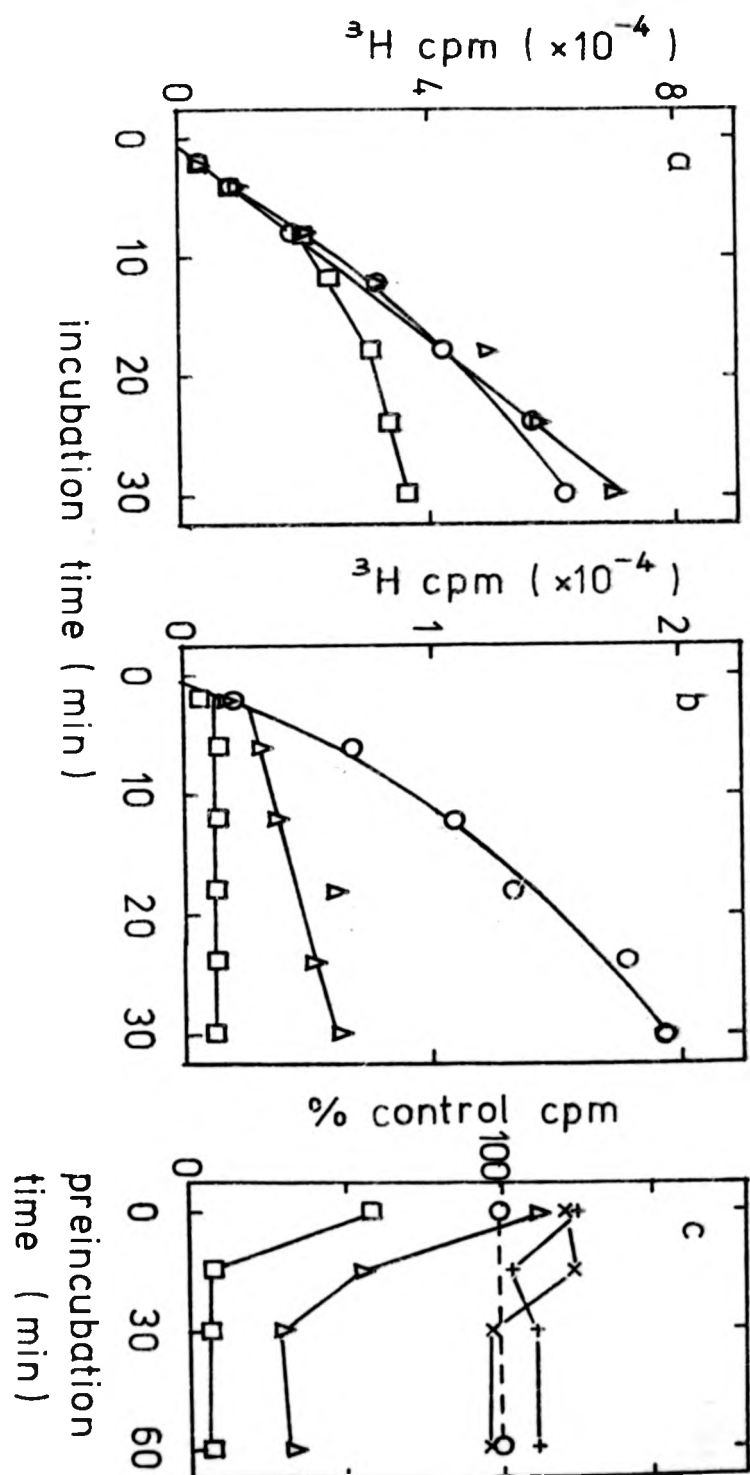
(a) Kinetics of protein synthesis with no preincubation

(b) Kinetics of protein synthesis after 60 min preincubation

(c) 30 min incorporations after various times of preincubation, expressed as percentages of the relevant control.

- control
- Δ 11  $\mu$ M poly rA.rU.rU
- 1  $\mu$ M poly rI.rC
- + 1  $\mu$ M poly rI
- × 1  $\mu$ M poly rC.





experiment in the lysate, adding poly rA.rU.rU in an 11-fold molar excess (7-fold excess of base triples over base pairs) before the poly rI.rC.

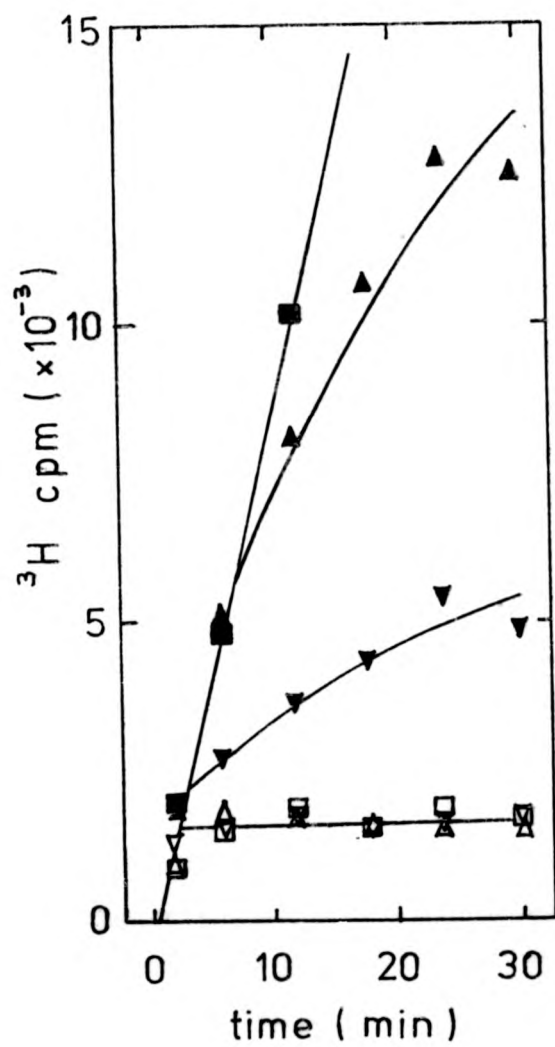
Whatever the time of addition of the poly rA.rU.rU relative to the poly rI.rC, there was no reduction in the poly rI.rC inhibition (Fig.10). If the binding of tsRNA to the lysate receptor were as strong as to the induction specific cell surface receptor, this protocol might have been expected to reduce the dsRNA inhibition.

FIG.10. Effect of poly rA.rU.rU on the inhibition due to poly rI.rC.

Reticulocyte lysate, with hemin added, was incubated alone or with poly rA.rU.rU at 11  $\mu$ M final concentration. If required, poly rI.rC was added to 1  $\mu$ M at 30 min, or poly rA.rU.rU to 11  $\mu$ M at 40 min. All incubations were continued to 60 min when other components for protein synthesis were added, including  $^3\text{H}$  leucine to 25  $\mu$ M and 50  $\mu\text{Ci/ml}$ . 5  $\mu\text{l}$  aliquots were taken as required for acid precipitation. The control was approximately linear up to 20 min, and then reached a plateau at 23000 cpm.

time(min)	0	30	40	
addition	none	none	none	■
	none	rI.rC	none	□
	none	none	rA.rU.rU	▲
	none	rI.rC	rA.rU.rU	△
	rA.rU.rU	none	none	▼
	rA.rU.rU	rI.rC	none	▽

Addition of poly rA.rU.rU at 10, 20, or 30 min also made no difference to the inhibition by poly rI.rC added at 30 min (data not shown).



#### 4.5. RELATIONSHIP BETWEEN INHIBITION AND MOLECULAR WEIGHT OF POLY rI.rC

A commercial "size kit" of poly rI and poly rC of different average lengths has been used extensively in interferon research. When annealed together to form short (2.5 S poly rI with 3.1S poly rC), intermediate, or long (12.5 S poly rI with 13.2 S poly rC) duplexes, interferon induction was reduced as molecular size was decreased (Stewart and de Clercq 1974). The effect of these duplexes on the lysate was determined.

Fig. 11 shows the kinetics of inhibition and dose-response curves for short, intermediate and long duplexes. Clearly, the difference in inhibitory effect depends on the concentration used. Above  $10^{-8}$  to  $10^{-7}$  M, all duplexes inhibit. Three independent experiments were performed to estimate the 50% inhibitory concentrations of the shortest and longest duplexes (Table 3). Somewhere between 5 and 50 times as much short dsRNA as long dsRNA is required for a given inhibitory effect.

Table 4 shows the results of an experiment using all six different size duplexes each added at 1 nM. Apart from the low inhibition due to the hybrid of 10.6 S poly rI with 10.5 S poly rC, which could be due to degradation of one of the strands, there is a general decrease in inhibition as size is reduced.

dsRNA molecules can also be constructed by annealing high molecular weight poly rI with low molecular weight poly rC, and vice versa. When annealed in equimolar concentrations of bases, the expected structures consist of duplexes in which one strand has occasional breaks. In the lysate, it appears that the integrity of the poly rI strand is more important than the integrity of the poly rC strand (Fig. 12).

Two questions arise from this work. Firstly, is the inhibitory effect of low molecular weight dsRNA due to contamination with high molecular weight material? Secondly, does the loss of biological activity as the size of both strands, and particularly the poly rI strand, is reduced, correlate with changes in stability?

FIG.11. Kinetics of inhibition of protein synthesis by different sizes of poly rI.rC.

Hybrids were made from different size classes of poly rI and poly rC. Dilutions were made as required, and preincubated in the reticulocyte lysate at 30°C for 30 min. Other components for protein synthesis were added, including  $^3\text{H}$  leucine at 80  $\mu\text{M}$  and 50  $\mu\text{Ci/ml}$ , and incubation continued. 5  $\mu\text{l}$  aliquots were removed as required for acid precipitation.

- control
- × 0.1 nM poly rI.rC
- 1 nM
- △ 10 nM
- ▽ 100 nM

- (a) The poly rI.rC was made from 2.5S poly rI and 3.08S poly rC
- (b) The poly rI.rC was made from 6.13S poly rI and 5.94S poly rC
- (c) The poly rI.rC was made from 12.5S poly rI and 13.2S poly rC

The control line from part (a) is also shown in parts (b) and (c), which are plotted with the ordinate expanded 2-fold.

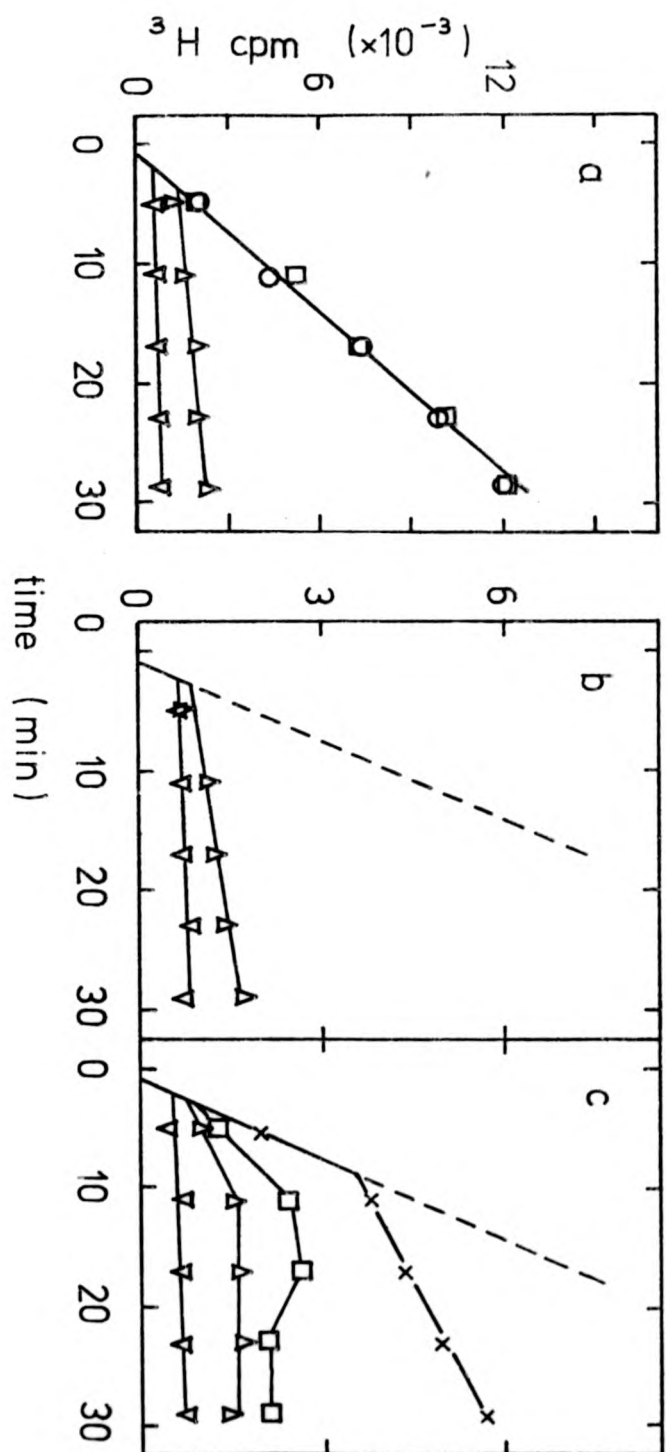


TABLE 3. Dependence of 50% inhibitory concentration  
on size of dsRNA.

poly rI.rC size		50% inhibitory concentration			
poly r.I S value	poly rC S value	(log molarity)			
		expt.no. I	II	III	
"short"	2.5	3.08	-8.4	-8.3	-8.4
"long"	12.5	13.2	-9.6	-9.0	-10.1
ratio "short" "long"		1.2	0.7	1.7	

Each experiment was performed as described in the legend to Fig. 11, except that two 5  $\mu$ l aliquots were taken after 30 min incubation with  $^3\text{H}$  leucine. Incorporation into acid insoluble material was plotted against the log concentration of dsRNA, and the 50% inhibitory concentration found by interpolation.



FIG.12. Relative importance of the poly rI strand for inhibition of protein synthesis by poly rI.rC.

Hybrids were formed by annealing together poly rI and poly rC of different size classes, at equimolar concentrations of nucleotides. Serial 5-fold dilutions were made and incubated with the reticulocyte lysate at 30°C for 30 min before adding the other components for protein synthesis, including <sup>3</sup>H leucine at 80 μM and 50 μCi/ml. After a further 30 min, two 5 μl aliquots were taken for acid precipitation. Incorporations are expressed relative to a control of 19400 cpm.

size of poly rI.rC		symbol	50% inhibitory concentration <sup>a</sup> (log molarity)
poly rI S value	poly rC S value		
2.5	3.08	○	-8.4
2.5	13.2	◐	-9.1
12.5	3.08	◑	-9.6
12.5	13.2	●	-9.6

a) 50% inhibitory concentration interpolated from the curves shown in the figure. These are not intended to be absolute figures, but give an indication of the relative importance of the poly rI and poly rC strands.

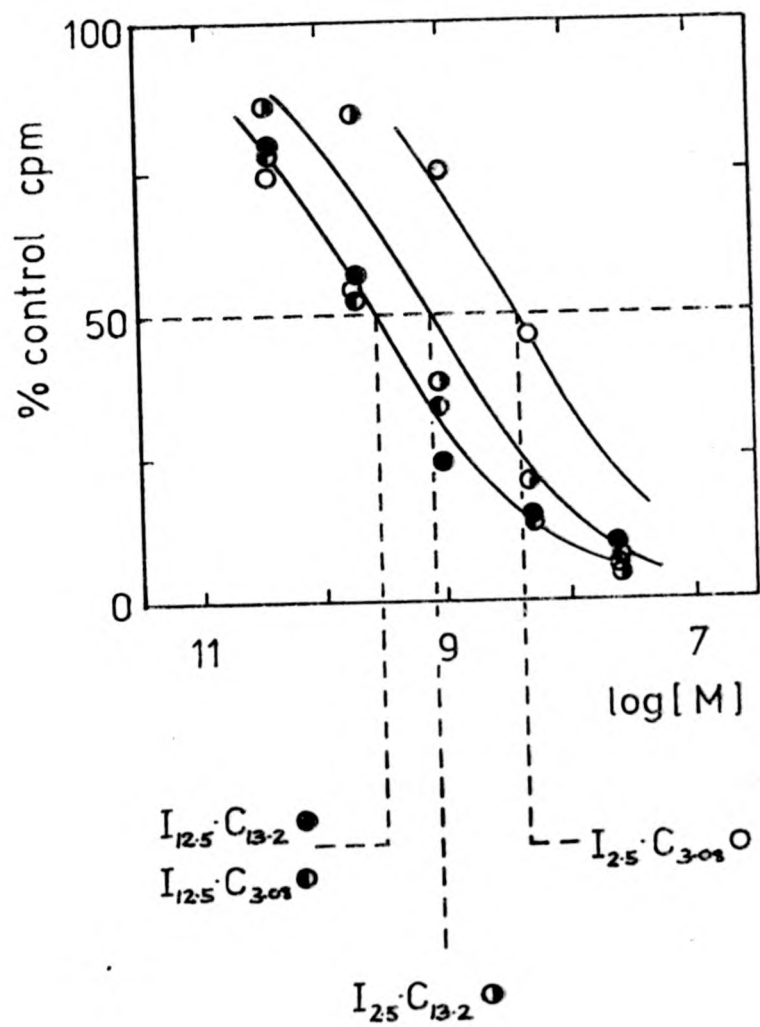


TABLE 4. Inhibition by different sizes of poly rI.rC  
assayed at 1 nM.

poly rI.rC size		% inhibition relative to control at 30 min
poly rI S value	poly rC S value	
2.5	3.08	4
4.39	3.88	6
6.13	5.94	18
7.94	8.20	63
10.6	10.5	34
12.5	13.2	65

Hybrids were prepared from different size classes of poly rI and poly rC, and added to the lysate at 1 nM. After 30 min at 30°C, other components for protein synthesis were added, including <sup>3</sup>H leucine at 80 μM and 50 μCi/ml. After a further 30 min, 5 μl aliquots were taken for acid precipitation. Control incorporation was 12100 cpm.

#### 4.6. SIZE HETEROGENEITY OF POLYNUCLEOTIDES

In view of the broad size range quoted by the manufacturers for the "size kit" polynucleotides (see section 2.5.1), the sedimentation characteristics of the poly rI preparations were determined (Fig. 13). These gradients were run under non-denaturing conditions, so reliable S values cannot be found. However, it is clear that only very small amounts (maybe 1-2%) of the "2.5 S" pieces sediment at 8 S or more, that is, are large enough to inhibit the lysate at 1 nM (Table 4). As diffusion could be significant and the gradients were heavily overloaded, this estimate is likely to be an upper limit. It thus seems probable that the inhibition caused by the short dsRNA molecules at high concentration (Table 3) is not caused by contamination with long molecules. However, it was desirable to repeat the inhibition experiments with less polydisperse preparations.

Sucrose gradients were found to be unsuitable for further fractionation of the poly rI preparations, because of the long times of centrifugation required. When poly rC was radioiodinated and fractionated on 3% or 7.5% polyacrylamide gels, good separation of low molecular weight pieces was obtained, but attempts to electrophorese the RNA from the gel slices and onto DEAE paper, followed by elution and concentration, were unsuccessful, and introduced problems of degradation and yield. These attempts were not pursued further. It might have been more profitable to fractionate the poly rI.rC than its constituent single strands, because dsRNAs show good resolution on gel electrophoresis (Shatkin et al 1968). However, the problem of recovery from the gel slices would have remained.

FIG.13 Sedimentation of poly rI on sucrose gradients.

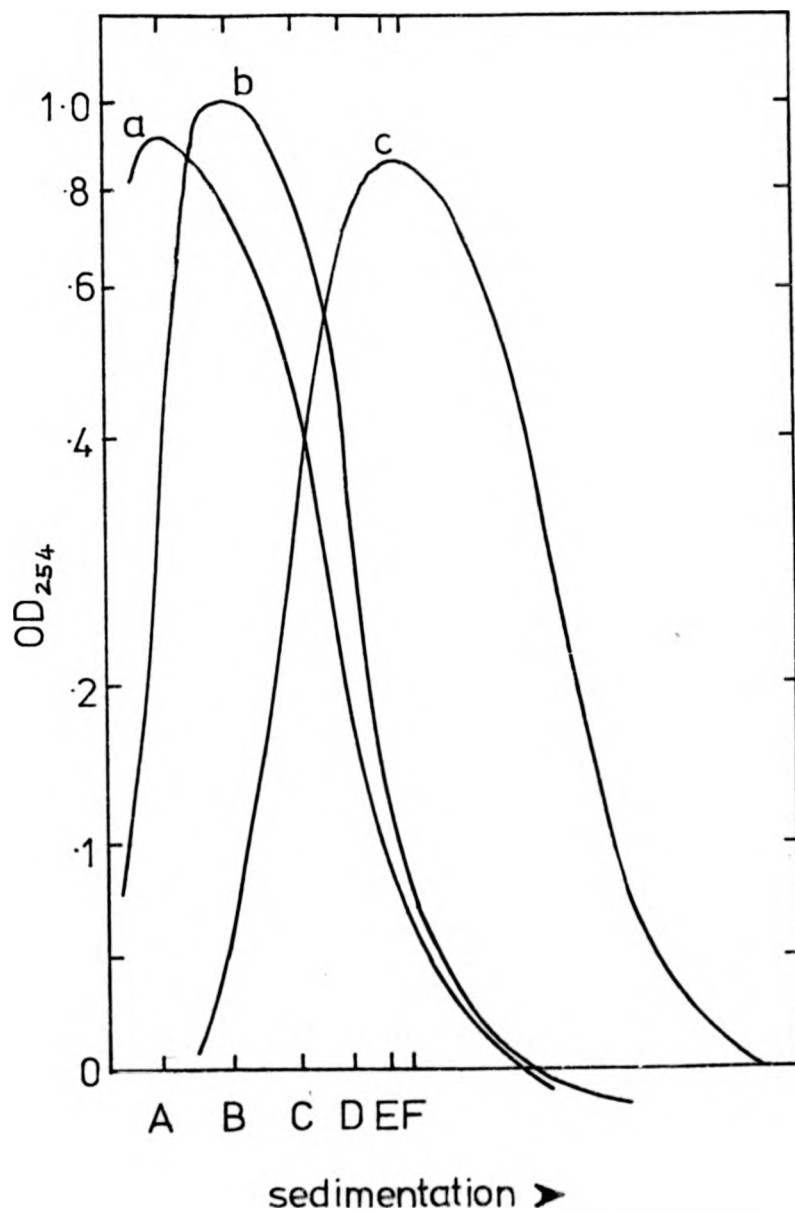
Poly rI pieces of different nominal lengths (Table 1) were centrifuged through 12 ml 5-30% W/W sucrose gradients in the MSE 6 x 14 ml rotor at 40000 rpm for 24 hr, maintained at 2°C. The gradients were made from 5% W/W and 30% W/W sucrose dissolved in TNE (section 2.4.1), and were convex, but not isokinetic. After centrifugation the gradients were unloaded from the top and pumped through the Uvicord. Percent transmission was converted to optical density and the optical density of some of the fractions checked in the SP600.

Three of the six profiles are shown:-

- (a) "2.5S" poly rI, 17 OD<sub>254</sub> units
- (b) "4.39S" poly rI, 14 OD<sub>254</sub> units
- (c) "12.5S" poly rI, 16 OD<sub>254</sub> units

and the markers are the peak fractions of these, and other gradients:-

- A, "2.5S"; B, "4.39S"; C, "6.13S"; D, "7.94S"; E, "10.6S";
- F, "12.5S".



#### 4.7. VARIATION OF CHEMICAL PROPERTIES WITH SIZE OF dsRNA

Low activity of short dsRNA molecules in the lysate assay could be due to a reduction in stability of the double-stranded structure. Stability was assessed by two methods - by following the optical melting curve, and by determining resistance to nuclease digestion.

When spectra of solutions of the duplexes, at either 25 or 85°C, were scanned from 210 to 310 nm, no differences were detected. However, when the optical density at 250 nm was monitored over this temperature range, significant differences were evident (Fig. 14a). The shorter duplexes showed a lower melting temperature ( $T_M$ ), lower hypochromicity and broader melting transition, than the longer duplexes. The breadth of the melting transition implies the presence of a population of molecules of different  $T_M$ s, that is, of different stabilities.

A similar analysis was performed on the hybrids made by annealing long strands with short (Fig. 14b). The poly rI strand length was found to be more important than the poly rC strand length for structural stability in agreement with their relative importance for inhibiting protein synthesis (Fig. 12).

Selected duplexes were also compared in terms of their resistance to ribonuclease digestion. The conditions were chosen to degrade poly rI and poly rC but not high molecular weight poly rI.rC (see section 3.2). The assay does not distinguish degradation of the poly rI strand from degradation of the poly rC strand, because digestion products of both strands contribute equally to the acid soluble optical density.

Table 5 shows that the shorter duplex is more sensitive to nuclease degradation than longer ones, which implies the existence of free single strands. These could either be present free in solution, or as local regions of instability within the double helix (Teitelbaum and Englander 1975). The results do not distinguish these mechanisms, but either explanation implies that the short duplexes are less stable. An alternative interpretation

FIG.14. Dependence of melting profile of poly rI.rC on  
molecular weight

Hybrids were formed using poly rI and poly rC of various sizes,  
and diluted in PBS (0.15 M monovalent cations) to an  $OD_{250}$  of  
0.4 to 0.5. Melting curves were followed at 250 nm as described  
(section 2.5.3), and increases in  $OD_{250}$  converted to percentages  
of the starting  $OD_{250}$ . Each curve is displaced relative to the  
others by 15%, for clarity.

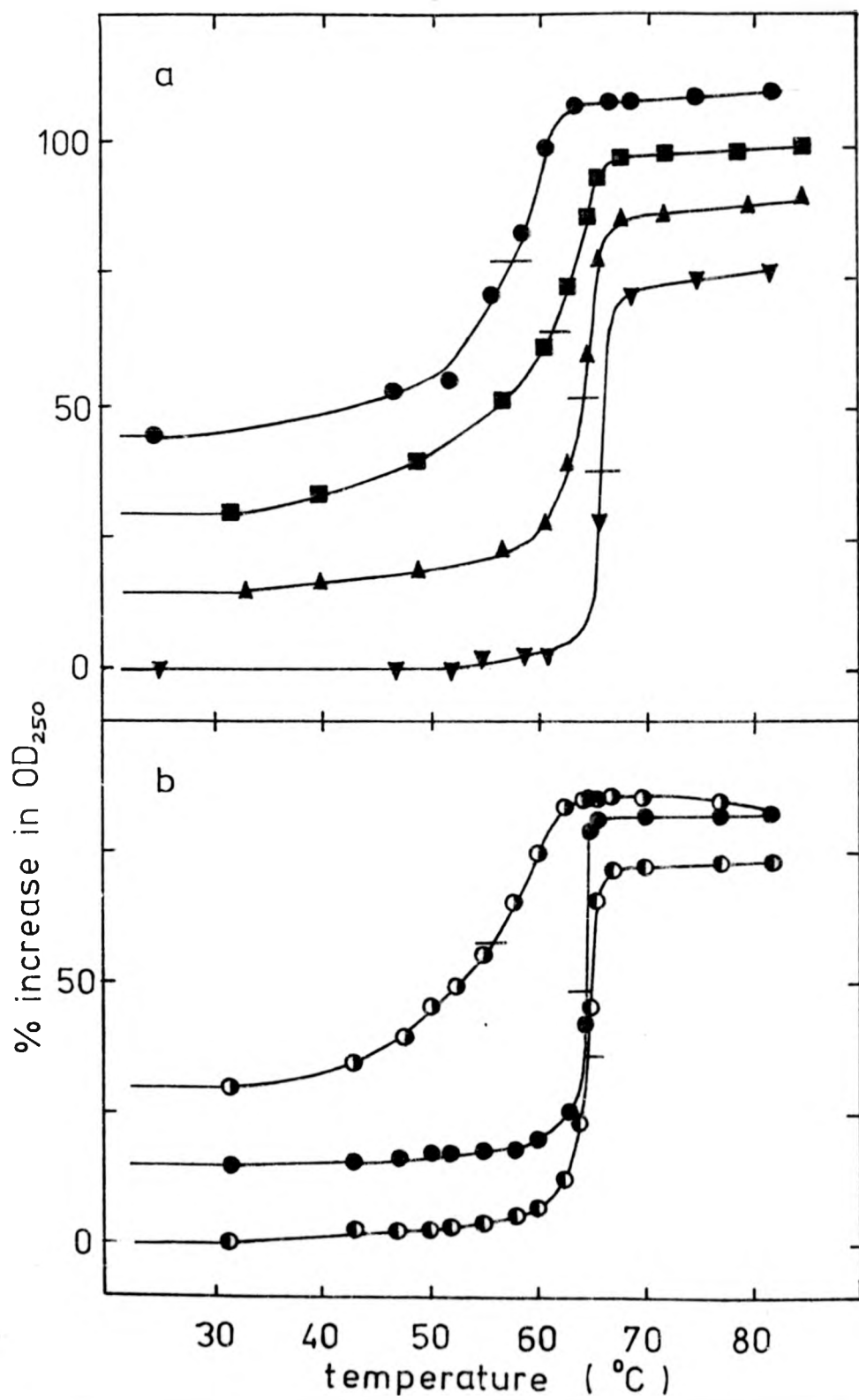
(a) Hybrids made from poly rI and poly rC of similar sizes

⊙	2.5S poly rI and 3.08S poly rC (melting temperature 58.0°C)	
■	4.39S	3.88S 63.5°C
▲	6.13S	5.94S 64.5°C
▼	12.5S	13.2S 66.0°C

(b) Hybrids made from poly rI and poly rC of different sizes.

⊙	2.5S poly rI and 13.2S poly rC (melting temperature 55.5°C)	
●	12.5S	13.2S 64.5°C
○	12.5S	3.08S 64.5°C





is that a few breaks in a short strand may produce more acid-soluble fragments than a proportional number of breaks in a long strand. However, this effect should be negligible, provided that the smallest strand length considered is many times the maximum size for acid solubility, as is the case in these experiments.

TABLE 5: Variation of chemical and biological properties of poly rI.rc with molecular size

poly rI S value	poly rC S value	melting temp. (°C)	% ribonuclease sensitivity <sup>c</sup>	toxicity <sup>d</sup>	interferon induction (% control yield) primed <sup>e</sup>	unprimed <sup>f</sup>	50% inhibition of protein synthesis (log molarity) <sup>g</sup>
12.5	13.2	64.5 <sup>a</sup> to 66.0 <sup>b</sup>	11	++	100	100	-9.0 to -10.1 <sup>h</sup>
6.13	5.94	64.5 <sup>b</sup>	15	++	100	70	ND <sup>j</sup>
4.39	3.88	63.5 <sup>b</sup>	35	+	20	30	ND
2.5	3.08	58.0 <sup>b</sup>	63	-	< 3	< 5	-8.3 to -8.4 <sup>h</sup>
12.5	3.08	64.5 <sup>a</sup>	ND <sup>j</sup>	++	75	120	-9.6 <sup>i</sup>
2.5	13.2	55.5 <sup>a</sup>	ND	-	< 3	< 5	-9.1 <sup>i</sup>

Hybrids formed from poly rI and poly rC of various sizes were assayed for various chemical and biological characteristics.

- a) From Fig. 14b . b) From Fig. 14a c) Ribonuclease sensitivity assayed according to the procedure described (section 2.5.4) and normalised with respect to complete digestion by ribonucleases in low salt conditions. d),e) and f) data from Stewart and de Clercq (1974) d) toxicity of 50 µg/ml poly rI.rc for Lpa cells treated with 100 u/ml interferon. - , no toxicity +, up to 25% and ++, up to 50% destruction of monolayer. e) Interferon titre induced in Lpa cells primed with 100 u/ml interferon and induced with 50 µg/ml poly rI.rc. f) Interferon titre induced in Lpa cells by 10 µg/ml poly rI.rc complexed with 100 µg/ml DEAE dextran. g) 50% inhibitory concentration in the reticulocyte lysate. h) From Table 3. i) From Fig. 12. j) ND = not determined.

#### 4.8. DISCUSSION

For a polynucleotide to be capable of inducing interferon in normal or primed cells, and to cause cytopathic effects in interferon-treated cells, it must have a stable secondary structure, high molecular weight, and 2' hydroxyl groups on both strands (de Clercq 1974; Stewart et al 1973a; Stewart and de Clercq 1974). These requirements have now been shown to apply for another biological activity - the inhibition of protein synthesis in a cell-free system from rabbit reticulocytes. This conclusion has since been confirmed by Hunter et al (1975), who studied a variety of bacteriophage mycophage and viral RNAs as well as some homopolymers.

The use of a homologous series of homopolymers (formed from poly rI, poly rC, poly dI and poly dC) has enabled the analysis of each parameter independantly of base composition. ssRNAs, dsDNA and RNA.DNA all inhibit poorly and with different kinetics from dsRNA (Fig. 8). The tsRNA poly rA.rU.rU (Michelson et al 1967; de Clercq et al 1974; de Clercq et al 1975b), is not inhibitory, and does not prevent the inhibition by dsRNA (Fig. 9,10). It is not impossible, however, that conditions could be found where tsRNA could prevent the effects of dsRNA, so this result should not be taken as evidence that the induction specific receptor and the lysate receptor recognise different characteristics of the polynucleotide structure.

The results using different length duplexes show that short duplexes are less effective than long ones, but even the shortest duplex tested is inhibitory at high concentrations (Table 3, Fig. 12). The question of the minimum size for inhibition, discussed by Hunter et al (1975), may not be meaningful if it depends on the concentration at which the dsRNA is added. Moreover, the increase of inhibition with size of dsRNA can be rationalised on the basis of the requirements for a stable secondary structure and for resistance to nucleases. As the molecular weight of the poly rI strand, and to a certain extent the poly rC strand, is reduced, so is the melting temperature and ribonuclease resistance of the hybrid (Table 5). This correlates with a reduction in all the biological activities studied (data

presented in Table 5 from Stewart and de Clercq 1974; de Clercq 1974), and suggests that the absolute size of the hybrid is immaterial, and that what is important is structural stability. Indeed, any receptor capable of interacting with, say, 100 base pairs of dsRNA would probably need to be an aggregate of proteins, because individual proteins are only capable of binding to short stretches of double-stranded polynucleotides (Gilbert and Maxam 1973; Nathans and Smith 1975; Kornberg 1974). Such aggregates could exist on the cell surface (Edelman et al 1973), and could be important in mitogenesis (Dean et al 1972), but are difficult to envisage in a membrane-free lysate.

The degree of stability in question is not the difference between double strands and single strands, because all the duplexes used are stable at the assay temperature (Fig. 14). Rather it is a question of local perturbations in structure (de Clercq and Merigan 1971; Teitelbaum and Englander 1975), which could be significant in a situation where cellular proteins might selectively stabilise and destabilise different polynucleotides (Travers 1976; de Clercq et al 1975b).

On the basis of the limited survey presented here it appears that the lysate receptor recognises the same characteristics as the induction and toxicity - specific cell surface receptor. It would be interesting to extend this work further to establish fine-structure requirements. In particular, the poly  $c^7A.rU$  analogues which are inactive in induction (A.M. Bobst, personal communication 1976), and those batches of poly rI which have been reported to induce interferon without complexing to poly rC (E. de Clercq, personal communication 1975).

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SECTION 5  
CHANGES IN CELLS EXPOSED TO  
INTERFERON AND dsRNA

## 5. CHANGES IN CELLS EXPOSED TO INTERFERON AND dsRNA

### 5.1. INTRODUCTION

When interferon-treated cells are exposed to dsRNA, the titre of interferon produced may be altered - either being primed (Stewart et al 1971a) or blocked (Stewart et al 1971b; Friedman 1966) relative to the levels produced by control cells treated with dsRNA. In L929 cells, in the absence of DEAE dextran and with low interferon doses, only priming is important. Additionally, the cells may show a pronounced cytotoxic effect, not evident when control cultures are treated with dsRNA (Stewart et al 1972a). Such interferon-enhanced cytotoxicity is only exhibited by dsRNA (Stewart et al 1972a) and by vaccinia virus (Joklik and Merigan 1966). Other compounds tested are equally toxic for interferon-treated and control cells (Stewart et al 1973a). The toxicity of vaccinia virus may involve the dsRNA which is produced during virus multiplication (Stewart et al 1973a).

Toxicity and primed interferon production thus requires both interferon and dsRNA. The active component in the interferon preparation is probably the same as that which causes the antiviral effect (Stewart et al 1972a; Stewart et al 1973b), and the structural features recognized in the dsRNA molecule are the same for induction, primed induction, and toxicity (Stewart and de Clercq 1974). Indeed, the cytotoxic and priming effects are closely linked in temporal development, dose response, and time of exposure to dsRNA (de Clercq and de Somer 1974b) and complex manipulations are required to dissociate them (de Clercq and de Somer 1974b).

It is not clear what metabolic changes occur in cells as a result of exposure to interferon. The antiviral state (Dianzani and Baron 1975), the primed state (Stewart et al 1971b), and perhaps the increased sensitivity to the toxicity of dsRNA (de Clercq and de Somer 1974b; Stewart 1975), all develop rapidly. Although it seems that protein synthesis is required for the antiviral state (Levine 1964) but not for the primed state (Stewart et al 1971a), these results are complicated by the need to remove the



inhibitors during the challenge or interferon production period, and by the possibility of superinduction (Chany et al 1971). The metabolic requirements for the enhanced toxicity of dsRNA are unclear, presumably because of problems in interpreting a cytotoxic effect when inhibitors are present, but there is unpublished evidence (quoted by Stewart 1975) that protein synthesis is not required. In human cells enucleation prevents the antiviral state developing (Radke et al 1974), but both priming and toxicity are independent of the dosage of chromosome 21 (de Clercq et al 1975a), which is important for the antiviral state (Revel et al 1976).

In view of the pronounced changes seen after dsRNA treatment (Heremans et al 1976) it seemed profitable to investigate events occurring during this period, rather than during the interferon treatment period. This also had the advantage of built-in controls for non-specific effects of interferon or dsRNA treatment, because whereas any changes which were apparently interferon specific could be due to contaminants in the interferon preparation, and any changes which were dsRNA dependant could be due to binding or ionic effects, only those changes requiring both interferon and dsRNA would be implicated (either causally or coincidentally) in the cytotoxic and priming effects.

During preliminary characterisation of the toxic effect it became apparent that the treatment did not affect all cells equally, but a significant proportion of the cells remained viable. The biological basis for this phenomenon was investigated.

## 5.2. CHARACTERISTICS OF THE CYTOTOXIC EFFECT

Cultures of L929 cells grown as monolayers in plastic dishes or in glass vials showed a cytopathic effect when treated with 100-300 u/ml of interferon overnight, followed by poly rI.rC at 50 $\mu$ g/ml. Under these conditions about 50u of interferon were produced per 10<sup>6</sup> cells. Cytopathic effects and interferon could not be detected (less than 0.3u interferon/10<sup>6</sup> cells) when control cultures were treated with poly rI.rC or in cultures which had been treated with interferon but not with poly rI.rC.

The cytotoxic effect was routinely quantitated as follows. Cultures were grown to confluence in glass vials and treated with interferon for 15-17 hr. The cells were washed free of serum, incubated with poly rI.rC for 1 hr, and then washed and incubated for a further 2.5 hr, still in the absence of serum. Total protein remaining attached to the glass was then measured after washing to remove dead cells. In all experiments, triplicate interferon and poly rI.rC treated cultures were compared to three sets of controls: interferon but no poly rI.rC, and mock-treated cultures with or without poly rI.rC.

A typical assay is shown (Fig. 15a). The quantities of protein in the three sets of controls are similar, but the quantity in the treated cultures is reduced. The cytotoxic effect is defined as the proportional loss of protein when the treated cultures are compared with the average of the three sets of controls.

This assay method was compared with the conventional dye uptake procedure (Finter 1969), and counts of trypan blue excluding cells (Fig. 15a,b,c). The results of the dye uptake and cell counting procedures are comparable with the results reported by Stewart *et al* (1972a). Parallel cultures were used to assay interferon production (Fig. 15d), before staining with gentian violet to demonstrate the visible effects of interferon and poly rI.rC treatment (Fig. 15e).

The photomicrographs (Fig. 15f,g) show petri dish cultures from a separate batch of L929 cells. The interferon and poly rI.rC treated culture

FIG.15. Changes in L929 cells exposed to interferon and poly rI.rC

(a,b and c) Cultures of cells in glass vials were treated with interferon and poly rI.rC as described (section 2.2.7). After 3.5 hr, the cultures were washed 3 times with PBS and

(a) the protein remaining on the monolayer quantitated (sections 2.2.7 and 2.7.1); or

(b) the monolayers were incubated with 0.2 mg/ml neutral red, 20 mM tris HCl pH 7.4, 2mM  $MgCl_2$  in Earle's salts for 1 hr before washing twice with PBS and eluting the stain with 12 ml of 50% v/v ethanol, 110 mM acetic acid, 50 mM NaOH; or

(c) the cells were trypsinised and trypan blue excluding cells counted (section 2.2.9).

In each case the results shown are means of triplicate cultures treated with A, interferon and poly rI.rC; B, interferon alone; C, "mock-interferon" and poly rI.rC; and D, "mock-interferon" alone.

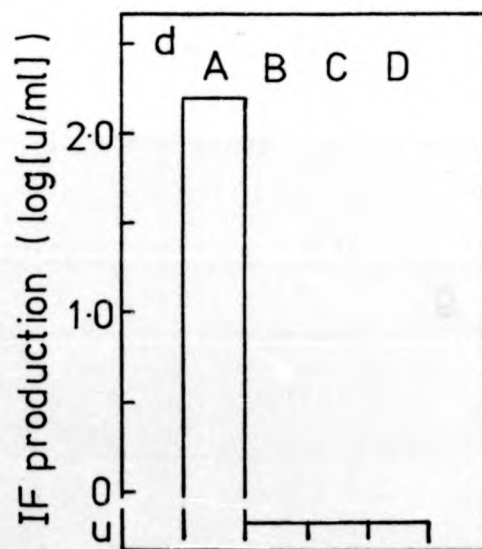
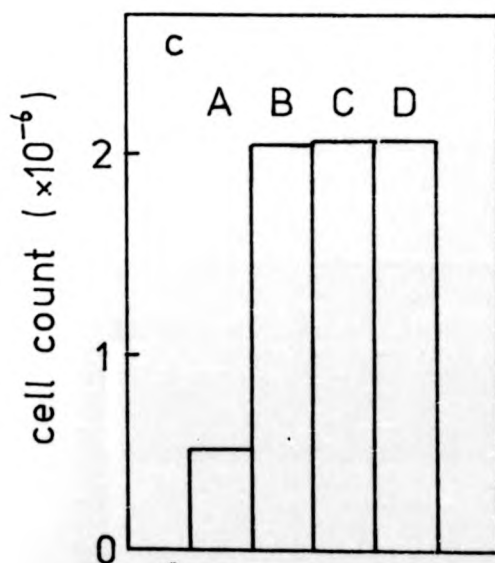
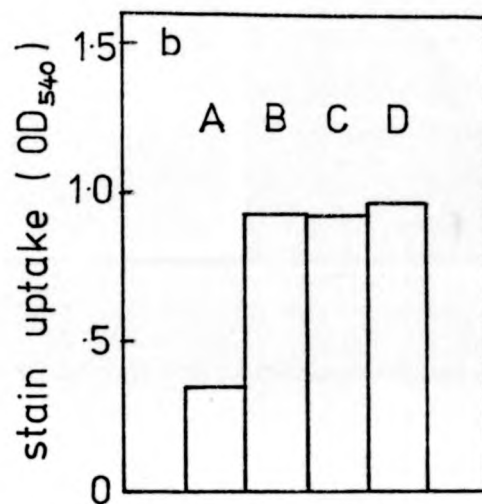
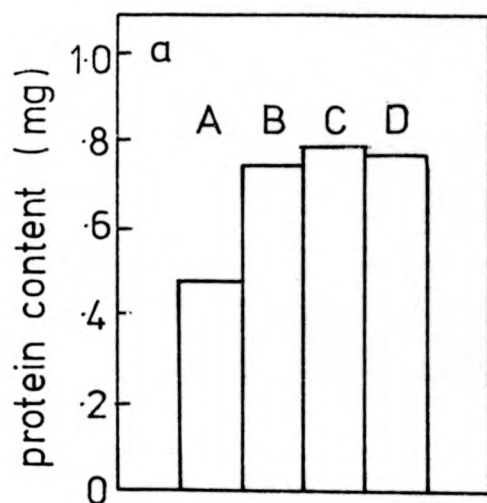
(a) shows the total protein content per culture, (b) the relative neutral red stain incorporation measured by  $OD_{540}$ , and (c) the total number of trypan blue excluding cells per culture.

(d and e) Cells seeded in 50 mm dishes were treated with interferon and poly rI.rC as required. The interferon produced between 1 and 24 hr after poly rI.rC was collected in 2 ml GMEM/2% CS, and titrated after pH2 treatment (sections 2.2.5 and 2.2.6). The interferon titre (d) and appearance of the gentian violet stained monolayers (e; section 2.2.6) are shown for A, interferon and poly rI.rC; B, interferon; C, "mock-interferon" and poly rI.rC; and D, "mock-interferon" treated cultures.

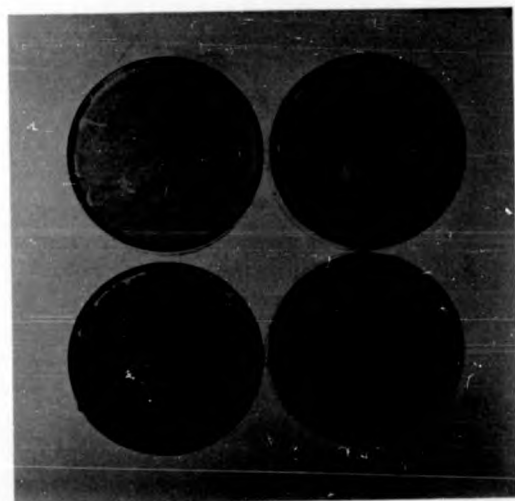
(f and g) Cells were treated with interferon and dsRNA (f) or "mock-interferon" and dsRNA (g) and photographed 3.5 hr after treatment (section 2.2.9). The medium was left on the cells for photography; if it had been removed and the cells washed, most of the rounded cells in (f) would have been lost.

protein content (mg)

1.40-61



e



f



g



shows the pronounced cytopathic effect evident 3.5 hr after poly rI.rC treatment, most of the rounded cells being readily removed by washing (Fig. 15f). On the other hand, the culture treated with poly rI.rC alone shows no cytopathic effects (Fig. 15g), and was identical with the other control cultures (not shown).



FIG. 16 Parallel loss of DNA and protein during cytotoxic effect.

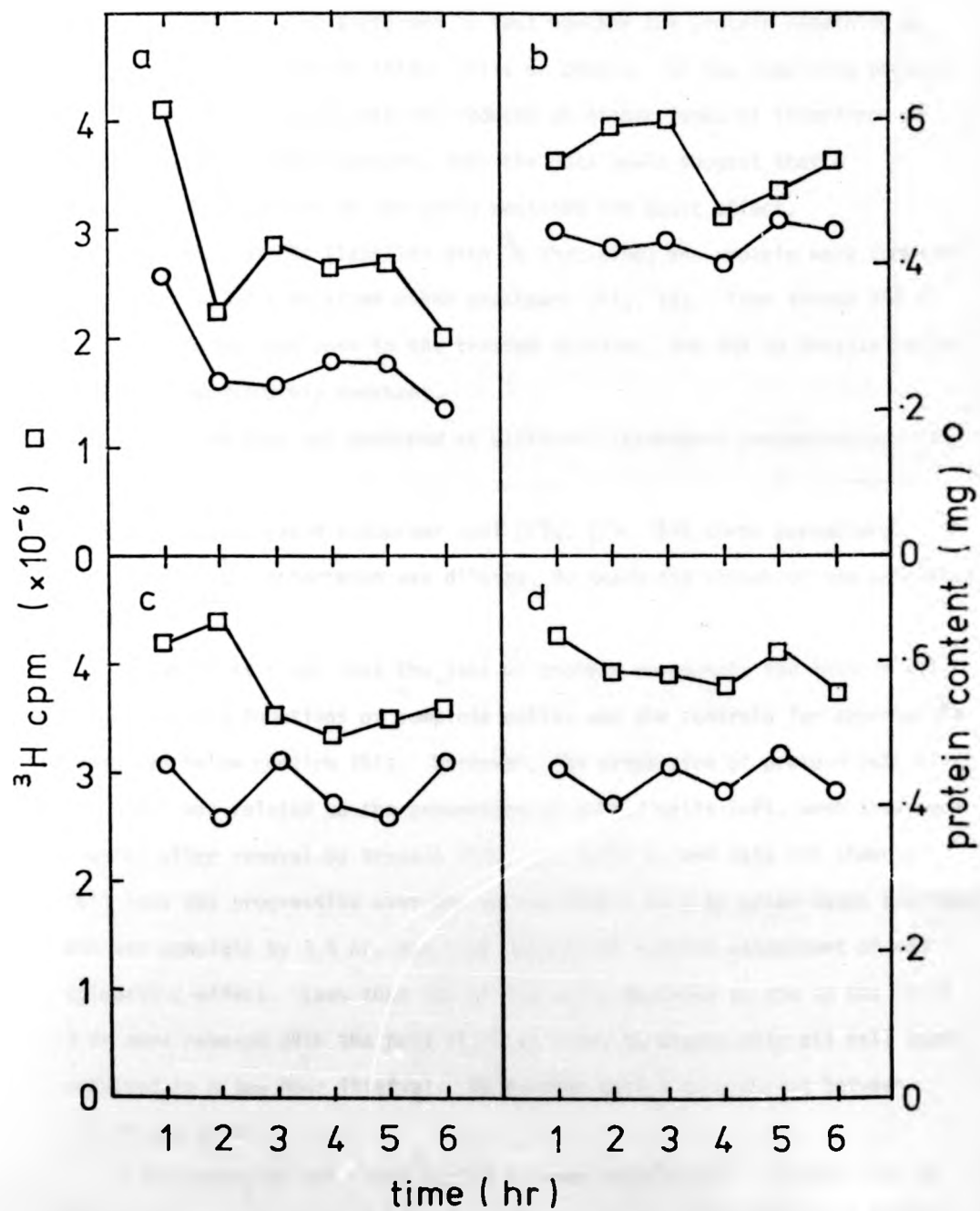
Cells were seeded in glass vials and labelled with  $^3\text{H}$  thymidine at 5  $\mu\text{Ci/ml}$  for 24 hr before treating with interferon and poly rI.rC as usual (section 2.2.7). At various times after poly rI.rC addition the cultures were washed three times with PBS, fixed with TCA, dried, and solubilised in 0.5 ml 1M NaOH and 50  $\mu\text{l}$  aliquots used for scintillation counting and for protein estimation.

□ DNA content, TCA insoluble  $^3\text{H}$  cpm per culture

○ protein content, mg protein per culture.

- (a) Cells treated with interferon and poly rI.rC
- (b) Cells treated with interferon.
- (c) Cells treated with "mock-interferon" and poly rI.rC
- (d) Cells treated with "mock-interferon".

$^3\text{H}$  cpm ( $\times 10^{-6}$ ) □





### 5.3. HETEROGENEITY IN THE RESPONSE OF CELLS TO THE TOXIC EFFECT

Experiments were performed to test whether the protein remaining on the monolayer represented intact cells or debris. If the remaining protein was in intact cells, and was not reduced at higher doses of interferon or dsRNA or after longer exposure, then the data would suggest that a significant proportion of the cells resisted the toxic effect.

The loss of DNA (labelled with  $^3\text{H}$  thymidine) and protein were compared over a period of 6 hr after dsRNA treatment (Fig. 16). Even though 35% of cellular protein was lost in the treated cultures, the DNA to protein ratio remained approximately constant.

When DNA loss was measured at different interferon concentrations (in the range 2-20000 u/ml) the results paralleled loss of protein synthesis capacity and aminoacid precursor pool (Fig. 17). All three parameters increased as the interferon was diluted, to reach the values of the untreated controls.

Thus it is clear that the loss of protein represents the loss of all components and functions of complete cells, and the controls for experiments presented below confirm this. Moreover, the proportion of protein left after treatment was related to the proportion of intact cells left, when they were counted after removal by trypsin (Fig. 15, Table 6, and data not shown). Cell loss was progressive over the period from 1 to 3 hr after dsRNA treatment, and was complete by 3.5 hr, the time chosen for routine assessment of the cytopathic effect. Less than 10% of the cells destined to die in the first 3 hr were removed with the poly rI.rC at 1 hr, so essentially all cell death occurred in a two hour interval. No further cell loss occurred between 3.5 hr and 24 hr.

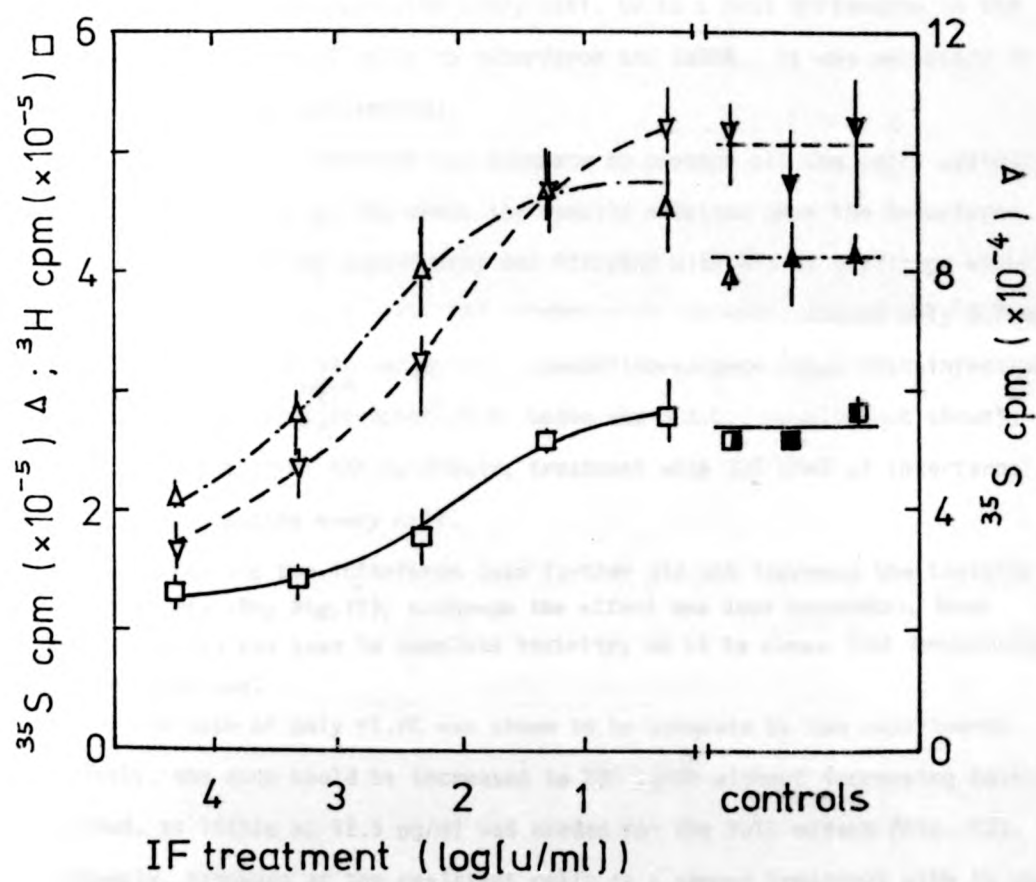
The extent of cell loss varied between experiments; the mean loss of protein after 300 u/ml interferon and 50 $\mu\text{g/ml}$  poly rI.rC was  $43.6 \pm 10.2\%$  (mean and standard deviation of 22 experiments, range 24% to 60%). Within

FIG.17. Loss of DNA and protein synthesis capacity.

Cells were seeded in glass vials in the presence of  $1 \mu\text{Ci/ml } ^3\text{H}$  thymidine and treated with different concentrations of interferon for 16 hr before washing and treating with poly rI.rC at  $50 \mu\text{g/ml}$ . At 3 hr the fluid were replaced with GMEM-M containing  $10 \mu\text{Ci/ml } ^{35}\text{S}$  methionine. After a further 30 min at  $37^\circ\text{C}$  the cultures were washed, fixed with TCA, and processed for TCA soluble and insoluble radioactivity (section 2.2.8).  $^{35}\text{S}$  to  $^3\text{H}$  spillover was negligible.

- DNA content,  $^3\text{H}$  cpm per culture
- ▽ methionine pool size, TCA soluble  $^{35}\text{S}$  cpm per culture
- Δ protein synthesis, TCA insoluble  $^{35}\text{S}$  cpm per culture

In each case the controls are: ■ ,  $10^{2.3}$  u/ml interferon but no poly rI.rC; ■ , "mock-interferon" and  $50 \mu\text{g/ml}$  poly rI.rC; ■ , "mock-interferon" and no poly rI.rC.



each experiment, however, the range of triplicates was usually of the order of 10% of the mean. The variation from experiment to experiment was not accounted for.

There is thus a significant proportion of cells (50-70% of cell protein) which resists the toxic effects of 300u/ml interferon and 50µg/ml poly rI.rC. This could be due to insufficient interferon or poly rI.rC being present to interact with every cell, or to a real difference in the response of individual cells to interferon and dsRNA. It was necessary to exclude the former explanation.

The dose of interferon was adequate to protect all the cells against virus infection. Fig. 18a shows the results obtained when the interferon used for the toxicity experiments was titrated with SFV as challenge virus. There was no detectable viral RNA synthesis at 300 u/ml, indeed only 0.7 u/ml were needed for the 50% end point. Immunofluorescence shows that infection at <sup>the</sup> ~~this~~ multiplicity <sup>used</sup> is total (K.B. Logan and J.A.C., results not shown), so, to prevent viral RNA synthesis, treatment with 300 u/ml of interferon must be protecting every cell.

Increasing the interferon dose further did not increase the toxicity of dsRNA (Fig. 18b; Fig. 17), although the effect was dose dependent. Even 20000 u/ml did not lead to complete toxicity, so it is clear that interferon was not limiting.

The dose of poly rI.rC was shown to be adequate by two experiments. Firstly, the dose could be increased to 200 µg/<sup>ml</sup> ~~44~~ without increasing toxicity - indeed, as little as 12.5 µg/ml was needed for the full effect (Fig. 19). Secondly, exposure of the resistant cells to a second treatment with 50 µg/ml poly rI.rC did not increase the toxic effect (Table 6). Additionally, limited toxicity is not due to the choice of poly rI.rC as the dsRNA, because Penicillium chrysogenum dsRNA (50 µg/ml) caused 44% loss of protein in an experiment in which poly rI.rC (50 µg/ml) caused 51% loss.

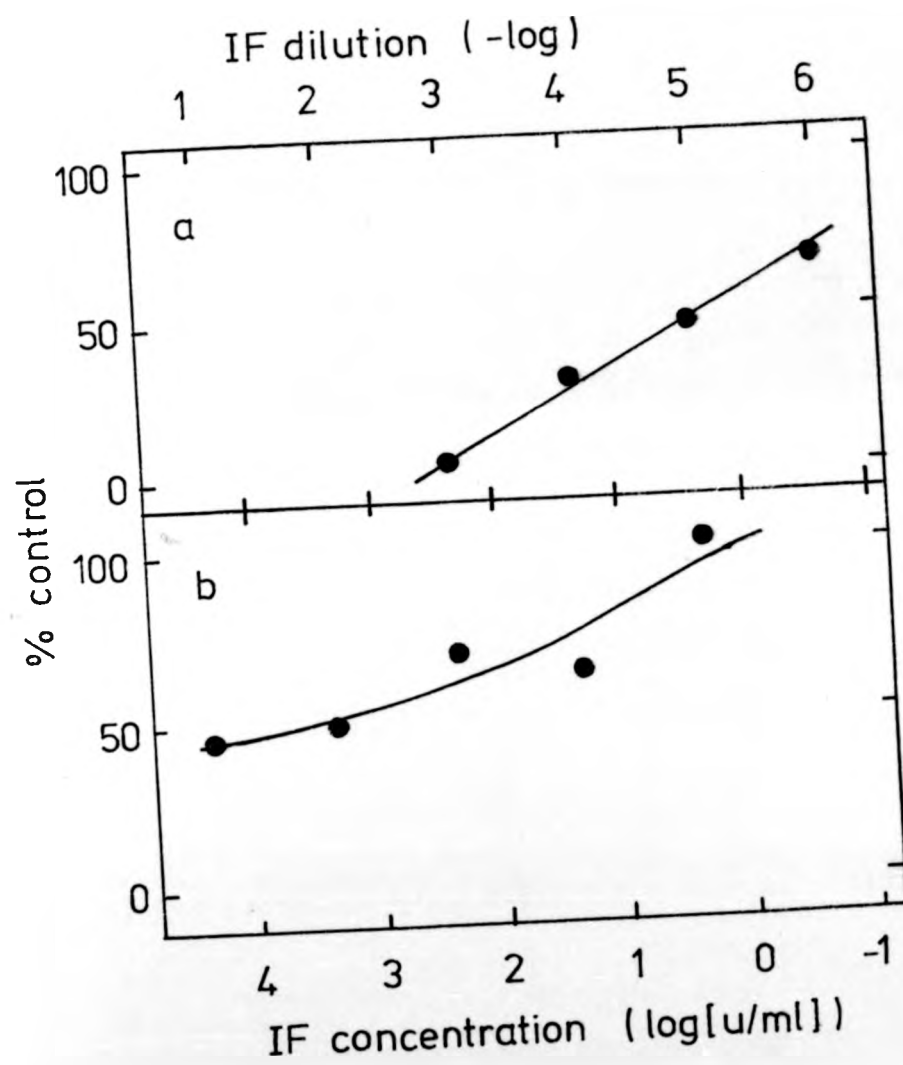
These results show that the resistant population is probably resistant because of a physiological difference between these cells and the sensitive

FIG.18. Effect of interferon concentration on antiviral effect and cytotoxic effect.

Cultures of L929 cells were treated with different dilutions of the same interferon stock. After 18 hr the cells were treated with 50  $\mu$ g/ml poly rI.rC as usual or challenged with SFV (section 2.2.5).

(a) Viral RNA synthesis as a percentage of controls treated with "mock-interferon". Comparison of this assay, and similar ones, with the antiviral effect of the NIH mouse standard interferon enabled calculation of the interferon concentration used, as given in the abscissa.

(b) Resistance to the cytotoxic effect, expressed as the protein remaining 3.5 hr after poly rI.rC treatment, relative to controls using  $10^{2.3}$  u/ml interferon but no poly rI.rC, "mock-interferon" and poly rI.rC, or "mock-interferon" alone.



cells, rather than a random effect due to limited supply of either interferon or dsRNA.



F

FIG.19. Effect of poly rI.rC concentration on cytotoxic effect.

Cells were treated with  $10^{2.3}$  u/ml interferon or "mock-interferon" for 18 hr and then with poly rI.rC at various concentrations. At 3.5 hr the cultures were washed and protein estimated as usual. The error bars show the range of duplicate cultures.

- interferon treated
- "mock-interferon" treated



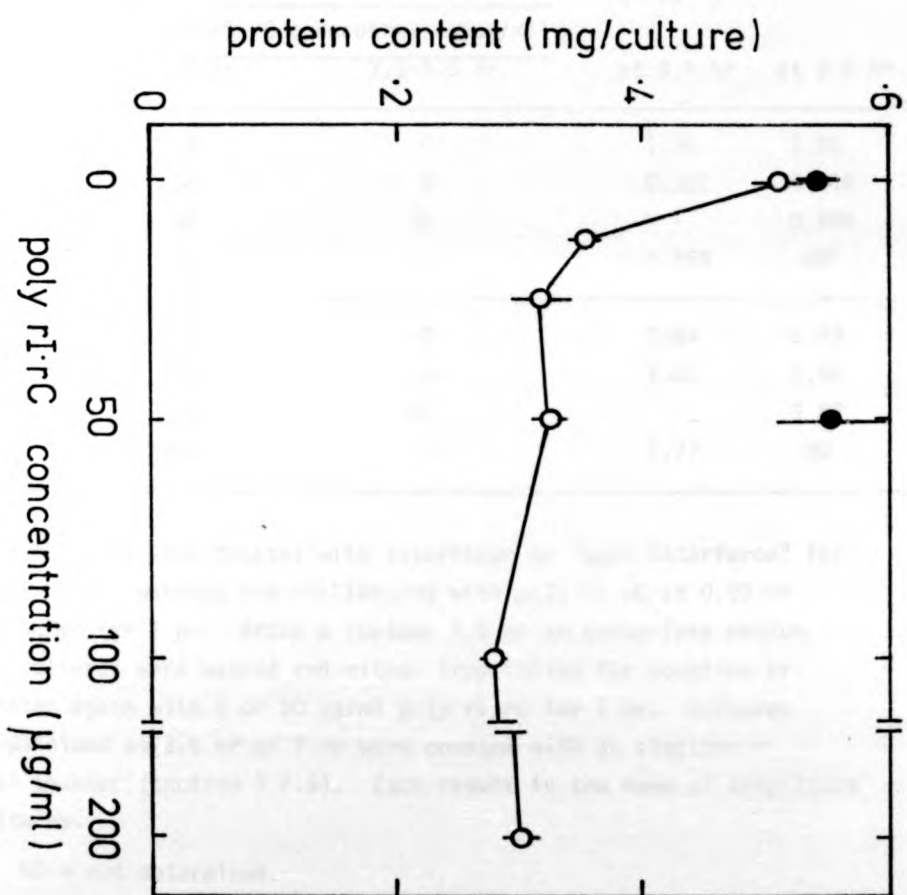


TABLE 6. Effect of second exposure to poly rI.rC on cell numbers.

treatment of culture			cell number per culture ( $\times 10^{-6}$ )	
interferon overnight	poly rI.rC concentration( $\mu\text{g/ml}$ )		at 3.5 hr	at 7.0 hr
	0-1 hr	3.5-4.5 hr		
$10^{2.3}$ u/ml	0	0	1.70	1.86
	50	0	0.322	0.312
	50	50	-	0.294
	100	0	0.256	ND <sup>a</sup>
"mock"	0	0	1.84	1.93
	50	0	1.82	1.99
	50	50	-	1.87
	100	0	1.77	ND

Cell cultures were treated with interferon or "mock-interferon" for 14 hr before washing and challenging with poly rI.rC at 0.50 or 100  $\mu\text{g/ml}$  for 1 hr. After a further 2.5 hr in serum-free medium the cultures were washed and either trypsinised for counting or treated again with 0 or 50  $\mu\text{g/ml}$  poly rI.rC for 1 hr. Cultures trypsinised at 3.5 hr or 7 hr were counted with an electronic cell counter (section 2.2.9). Each result is the mean of triplicate cultures.

a) ND = not determined.

#### 5.4. CHARACTERISTICS OF THE RESISTANT POPULATION

The population of cells resistant to the toxic effect is active in RNA and protein synthesis, provided that the results are normalised with respect to the total protein present.

In the same experiment that monitored DNA and protein loss (Fig. 16), RNA and protein synthesis rates and precursor pool sizes were measured (Fig. 20). There were some changes in the rate of protein synthesis in control cultures after 4 hr which were countered by corresponding changes in the pool size; with increased synthesis depleting the pool. This is consistent with the finding that methionine transport is passive (not inhibited by iodoacetic acid and sodium cyanide) and that inhibition of protein synthesis allows pool expansion (data not shown). These long term changes are thus probably actual changes in protein synthesis rates, and may represent recovery from the removal of serum. On the other hand, the parallel loss of acid soluble and insoluble radioactivity at early times in the treated cultures, does not represent the inhibition of protein and RNA synthesis, but the loss of cells (Fig. 16). The resistant cells are clearly still synthesising protein and RNA (see also Fig. 17).

The culture can be fractionated at 3.5 hr into resistant cells (present on the monolayer), sensitive cells (present in the washes) or total cells (complete culture, harvested without washing). When labelled with  $^3\text{H}$  uridine from 3 to 3.5 hr, the radioactivity in the total culture was due entirely to the resistant cells, and no radioactivity was found in the sensitive cells (Table 7). When the specific activity of the resistant cells is compared to that of the control cultures, the resistant cells are found to have been synthesising RNA at the control rate.

In any of the metabolic parameters studied, there were no significant differences between the resistant cells and control cells. Experiments were performed to gauge viability over longer times.

FIG.20. Changes in RNA and protein synthesis after poly rI.rC treatment.

Parallel cultures to those used before (Fig. 16) were treated with interferon and poly rI.rC as required. At various times after treatment the medium was removed and replaced with GMEM-M containing 5  $\mu\text{Ci/ml}$   $^{35}\text{S}$  methionine and 10  $\mu\text{Ci/ml}$   $^3\text{H}$  uridine for 30 min at  $37^\circ\text{C}$ , before washing with PBS and fixing in TCA. Samples of TCA soluble and insoluble material were prepared for scintillation counting (sections 2.2.8 and 2.7.2) and  $^3\text{H}$  counts corrected for  $^{35}\text{S}$  spillover.

Each graph shows:  $\circ$ , acid soluble  $^{35}\text{S}$  cpm;  $\bullet$ , acid insoluble  $^{35}\text{S}$  cpm;  $\square$ , acid soluble  $^3\text{H}$  cpm; and  $\blacksquare$ , acid insoluble  $^3\text{H}$  cpm, corrected for sample size to cpm per culture. Each point is the mean of duplicate cultures, and is plotted against the time of the start of the 30 min pulse.

- (a) interferon and poly rI.rC treated
- (b) interferon treated
- (c) "mock-interferon" and poly rI.rC treated
- (d) "mock-interferon" treated.

$^{35}\text{S}$  cpm ( $\times 10^{-4}$ ) 0

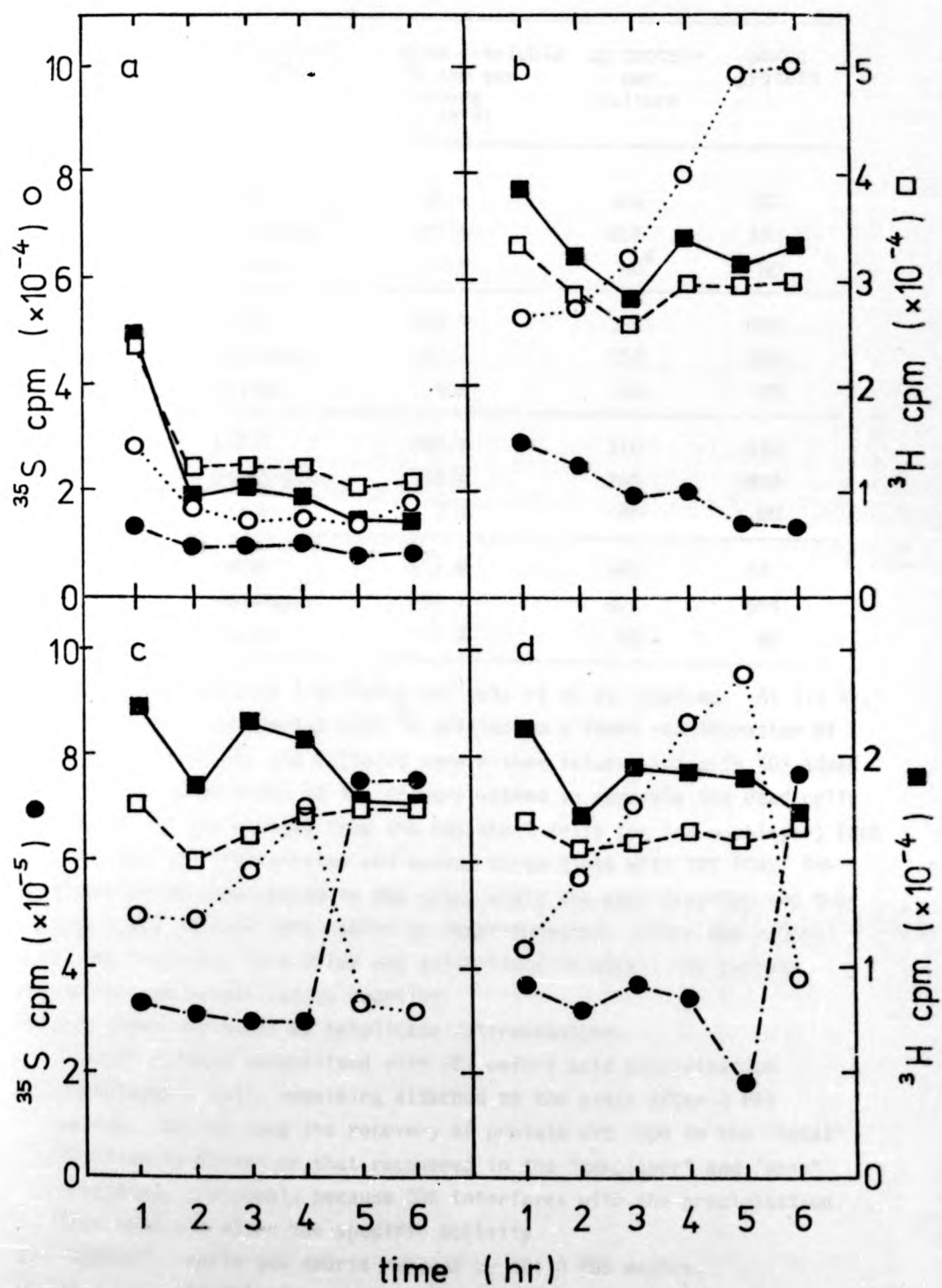


TABLE 7. RNA synthesis by resistant and control cells.

Treatment of cells	Fraction of culture	Acid insoluble $^3\text{H}$ cpm per culture ( $\times 10^{-3}$ )	$\mu\text{g}$ protein per culture	cpm/ $\mu\text{g}$ protein
interferon and polyrI.rC	total <sup>a</sup>	85.5	305	280
	monolayer <sup>b</sup>	100.6	202	501
	washes <sup>c</sup>	4.5	ND <sup>d</sup>	ND
interferon	total	209.7	305	687
	monolayer	241.1	353	684
	washes	8.8	ND	ND
"mock-interferon" and polyrI.rC	total	264.9	316	840
	monolayer	318.4	380	838
	washes	3.4	ND	ND
"mock-interferon"	total	217.6	340	647
	monolayer	255.1	370	694
	washes	7.2	ND	ND

Cells were treated with interferon and poly rI.rC as required. At 3.0 hr, the medium was supplemented with  $^3\text{H}$  uridine to a final concentration of 10  $\mu\text{Ci/ml}$ . At 3.5 hr the cultures were either solubilised with SDS, added to a final concentration of 1%, or were washed to separate the dead cells and debris (in the washes) from the resistant cells (on the monolayer). Each fraction was TCA precipitated and washed three times with 10% TCA. The monolayer cells were washed in the glass vial; the wash fraction and SDS-treated total culture were washed by centrifugation. After one ethanol wash, the fractions were dried and solubilised in alkali for protein estimation and scintillation counting.

Results given are means of triplicate determinations.

- a) "Total" culture solubilised with SDS before acid precipitation
- b) "Monolayer": cells remaining attached to the glass after 3 PBS washes. Notice that the recovery of protein and cpm in the "total" fraction is 81-84% of that recovered in the "monolayer" and "wash" fractions, presumably because SDS interferes with the precipitation. This does not alter the specific activity.
- c) "Washes" : cells and debris removed by the 3 PBS washes.
- d) ND = not determined.

When the protein content or cell number of treated cultures 24 hr after dsRNA treatment were compared with those 3.5 hr after treatment, the values were the same or slightly higher. These results were found in several experiments, whether or not serum was added at 3.5 hr (data not shown).

When the resistant cells were trypsinised at 3.5 hr and seeded in fresh medium, two different results were found, according to the seeding density.

In the experiment described in Table 8, the cells were seeded at high density ( $5-10 \times 10^4$  cells/cm<sup>2</sup>) in tissue culture flasks. The resistant cells took 3 days to reach confluence (about  $3.3 \times 10^5$  cells/cm<sup>2</sup>) whereas the control cultures took 2 days. This suggests that the resistant and control cells were equally viable, because about 30% fewer cells were seeded for the resistant cultures. In the next passage, resistant and control cultures took equal times to reach confluence and the numbers of cells harvested from each flask were similar.

On the other hand, when resistant and control cells were seeded at low density (about 50 cells/cm<sup>2</sup>), differences in their ability to form colonies were detected (Fig. 21). However, the inhibition of plating was interferon specific, and not dependent on both interferon and dsRNA, so might result from a growth inhibitory effect of interferon (Paucker *et al* 1962) or from a toxic contaminant in the interferon preparation. There was no more than a two-fold difference between interferon-treated cultures exposed to dsRNA and interferon-treated controls, as expected from the relative amounts of protein in the cultures (Fig. 21).

It thus seems that the cells surviving interferon and dsRNA treatment are capable of growth and division. By contrast, it is clear that by 3.5 hr after treatment the sensitive cells have lysed. When the washes (containing the remains of the sensitive cells) were centrifuged at low speed, only 50% of the protein sedimented, whereas more than 90% of the small



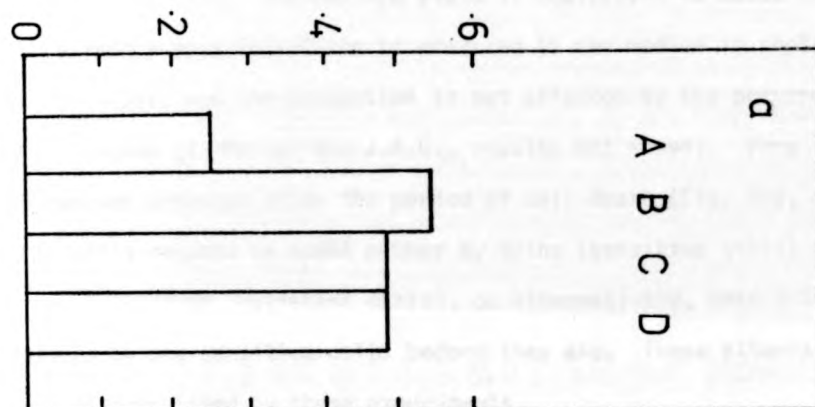
FIG.21. Plating efficiency of cells exposed to interferon and dsRNA.

Cultures of cells were treated with interferon and then with poly rI.rC, or under control conditions, as usual. After 3.5 hr, the cultures were washed three times with PBS and either fixed with TCA or trypsinised. Fixed cultures were used to determine the protein content. Trypsinised cells were diluted in GMEM/10% FBS and seeded into duplicate 50 mm petri dishes so that each dish contained  $1.6 \times 10^{-3}$  or  $1.6 \times 10^{-4}$  of the cells from one 25 mm vial culture. After 7 days at  $37^{\circ}\text{C}$  the dishes were drained, fixed with 4% v/v formaldehyde in 0.9% w/v saline, and stained with 1% w/v gentian violet in 20% v/v ethanol. All the colonies were about the same size. About 100 colonies were counted on the dish of suitable dilution, and the colony number corrected for the dilution to "colony forming units per culture".

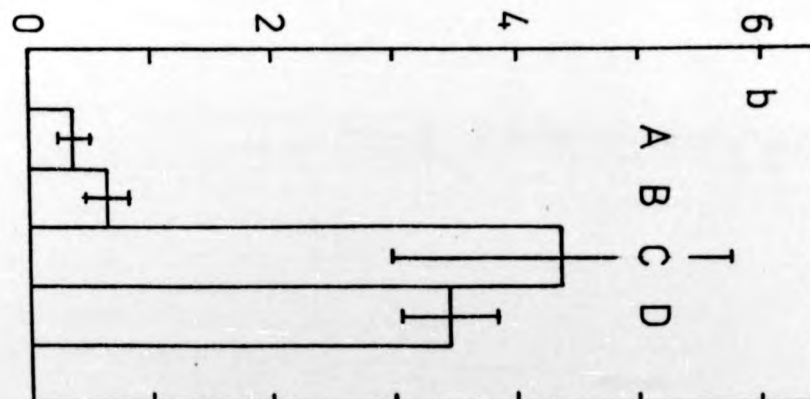
- (a) Protein content in mg protein per culture. Means of duplicates.
- (b) Colony forming units per culture. Results shown are means and standard deviations of 6 petri dishes per treatment (duplicate dishes from each of 3 initial cultures). In each case the treatment conditions were: A, interferon and dsRNA; B, interferon; C, "mock-interferon" and dsRNA; and D, "mock-interferon".



protein content ( mg/culture )



colony forming units  
per culture ( $\times 10^{-5}$ )



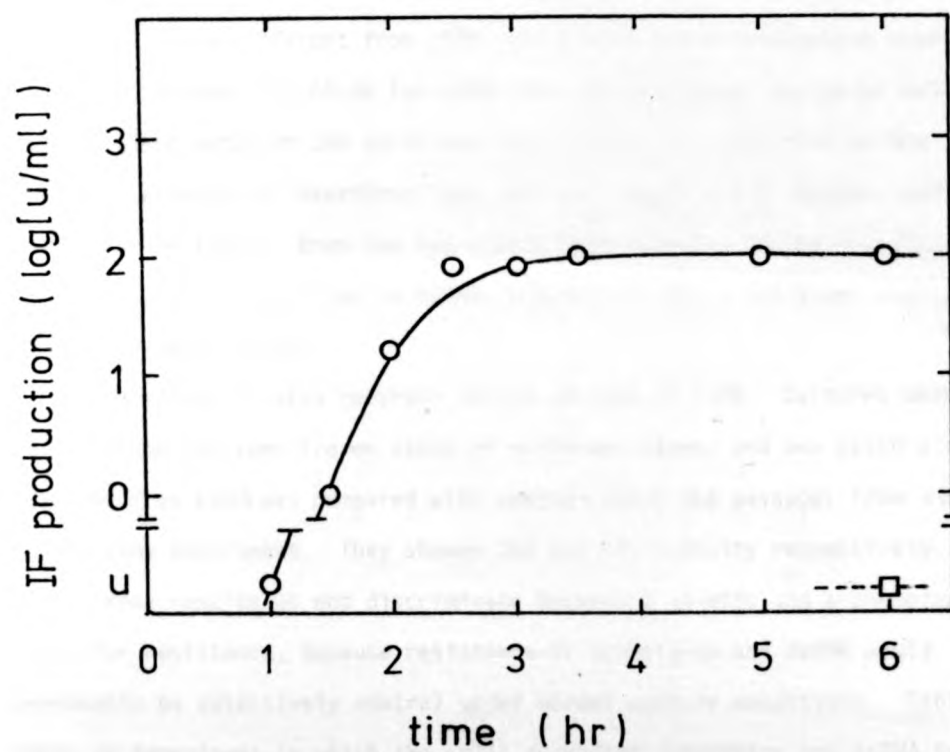
amount of protein released from control cultures was sedimented. When the washes were screened for large particles in an electronic cell counter, only 3% of the number of cells lost from the monolayer were countable (data not shown).

The time course of interferon production was determined in several experiments (Fig. 22). The maximum yield is equivalent to about  $50\text{u}/10^6$  cells. Slightly more interferon is produced if the medium is replaced at hourly intervals, and the production is not affected by the presence or absence of serum (J. Morser and J.A.C., results not shown). Very little interferon was produced after the period of cell death (Fig. 22), suggesting that the cells respond to dsRNA either by dying (sensitive cells) or by producing interferon (resistant cells), or alternatively, that interferon is produced by the sensitive cells before they die. These alternatives were not distinguished by these experiments.

FIG.22. Kinetics of interferon induction.

Petri dish cultures of L929 cells were treated with 200 u/ml interferon and 50  $\mu$ g/ml poly rI.rC as described (section 2.2.6). Interferon was collected in 2 ml medium and treated at pH2 before assay (section 2.2.5). Cumulative interferon production has been converted to international units per ml (u/ml) and is plotted on a log scale. "Undetectable" interferon production means that no antiviral effect was detected at  $10^0$  dilution, implying a titre of less than -1 log(u/ml). The maximum titre of  $10^2$  u/ml corresponds 200 u per petri dish culture, or about  $50 \text{ u}/10^6$  cells.

- ☐ primed cells
- ☐ unprimed cells



#### 5.5. BIOLOGICAL BASIS FOR THE TWO POPULATIONS

Attempts were made to determine the difference between the cells resistant to, and the cells sensitive to, treatment with interferon and poly rI.rC.

Firstly, the observation was repeated in another type of mouse L cells. LA9 is a mutant cell line isolated in 1964 (Littlefield 1964) which is widely used in cell fusion and genetic studies. Its genotype is probably very different from L929, LA9 having a mean chromosome count of 52-56 compared with 65-66 for L929 (the diploid number for mouse cells is 40). This batch of LA9 cells was only 2-fold less sensitive to the antiviral effects of interferon than L929 (H. Graves and A. Meager, personal communication 1976). When the two strains were compared in the toxicity assay, both were equally sensitive to dsRNA, suggesting that a resistant population is also present in LA9.

The effect is also constant during passage of L929. Cultures were started from the same frozen stock at different times, and one batch eleven passages from stock was compared with another batch two passages from stock, in the same experiment. They showed 38% and 45% toxicity respectively.

These results do not discriminate between a genetic and a phenotypic basis for resistance, because resistance to interferon and dsRNA would presumably be selectively neutral under normal culture conditions. Table 8 shows an experiment in which the cells surviving interferon and dsRNA treatment were cultured for 9 days and then tested for resistance to the toxic effect. There are no clear differences in toxicity dependent on both interferon and dsRNA, as would be expected if resistance was inherited as a simple genetic marker with a normal reversion rate (Meiss and Basilico 1972). There may be, however, some differences which are interferon dependent, perhaps because of a residual effect of interferon treatment (although the antiviral effect fully decays over the time interval involved, Paucker and Cantell 1963).

TABLE 8. Test for inheritance of resistance to the cytotoxic effect.

selection procedure	resistance of "selected" cells to cytotoxic effect (%)
interferon and poly rI.rC	72
interferon	70
"mock-interferon" and poly rI.rC	56
"mock-interferon"	56

Vial cultures were set up and treated as usual, with interferon and poly rI.rC or under control conditions. Parallel cultures showed about 30% loss of protein (i.e. 70% resistance), 3.5 hr after poly rI.rC treatment. At 3.5 hr the cultures were washed to remove debris and trypsinised. The cells from the duplicate vials corresponding to each treatment were pooled and transferred to a 25 cm<sup>2</sup> tissue culture flask. After 3 days the cells from each flask were passed to a 150 cm<sup>2</sup> flask, and after a further 4 days about  $50 \times 10^6$  cells were recovered from each and used to set up 25 mm vial cultures. These "selected" cells were used for assaying resistance to interferon and poly rI.rC. Treated cultures were compared with controls, 3.5 hr after treatment.

The Table shows the % resistance to interferon and poly rI.rC related to the past history of the cells.

Cells in culture show phenotypic variation due to the cell cycle. In an unsynchronised culture, some cells will be at each stage of the cycle, but in confluent, resting, fibroblasts, most cells enter a stage called G0 (Holley 1975). As L929 cells are transformed it is possible that they lack this degree of growth control, and heterogeneity may exist even in confluent cultures.

The cytotoxic effect was observed in cultures synchronised by the double thymidine block procedure (Lee and Rozee 1970). Interferon was present at all times until the cultures were treated with dsRNA, thus ensuring that all cells had been exposed to interferon at all stages of the cycle. The only variable was the stage of the cycle at which dsRNA was added.

These cells have a doubling time of 17-18 hr and reach confluence at about  $1.5 \times 10^6$  cells/vial culture ( $3.3 \times 10^5$  cells/cm<sup>2</sup>) when they grow only slowly (results not shown). A preliminary experiment suggested that synchronising confluent cells would be unsuccessful, only a part of the cycle being completed. The experiment was thus performed using non-confluent cells, and required two controls - that the time of interferon treatment was not important, and that the toxic effect did not vary with cell density. Firstly, interferon treatment for 4 hr was just as effective as treatment for 24 hr (39% and 42% toxicity respectively). Secondly, there was only slight variation of toxicity with cell density (Fig. 23), ranging from 51% toxicity in sparse cultures to 36% in confluent cultures.

Any variation found in synchronised cells could thus be attributed to the cell cycle and not to the length of interferon treatment or to the cell density. The cell cycle was monitored by DNA synthesis rate (Fig. 24a) and mitotic index. The first S-phase peaked at about 2 hr, and the second sometime between 12 and 18 hr. Mitosis occurred between 6 and 8 hr. Throughout the cycle the cytotoxic effect lay within 10% of the unsynchronised



FIG.23. Cytotoxic effect measured at different cell densities.

Cells were seeded into 25 mm glass vials at  $2, 4, 6$  or  $8 \times 10^5$  cells/culture. After 2 days the cultures were treated with interferon for 16 hr and then with poly rI.rC, and the protein content measured 3.5 hr after treatment as usual.

(a) seeded at  $8 \times 10^5$  cells/culture, confluent when treated with poly rI.rC.

(b) seeded at  $6 \times 10^5$  cells/culture, not confluent

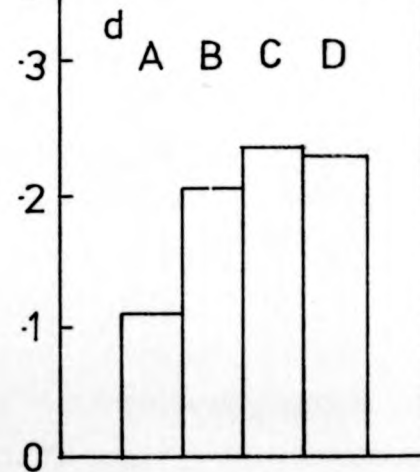
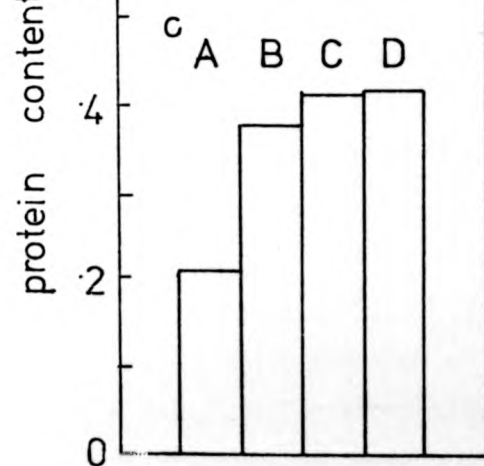
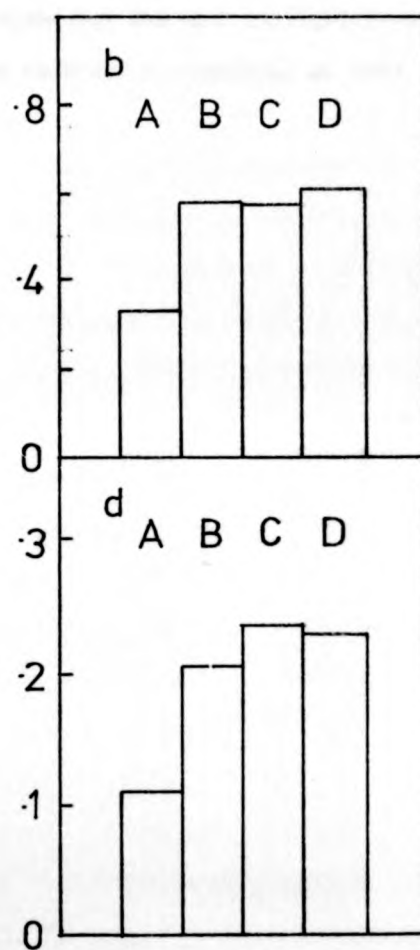
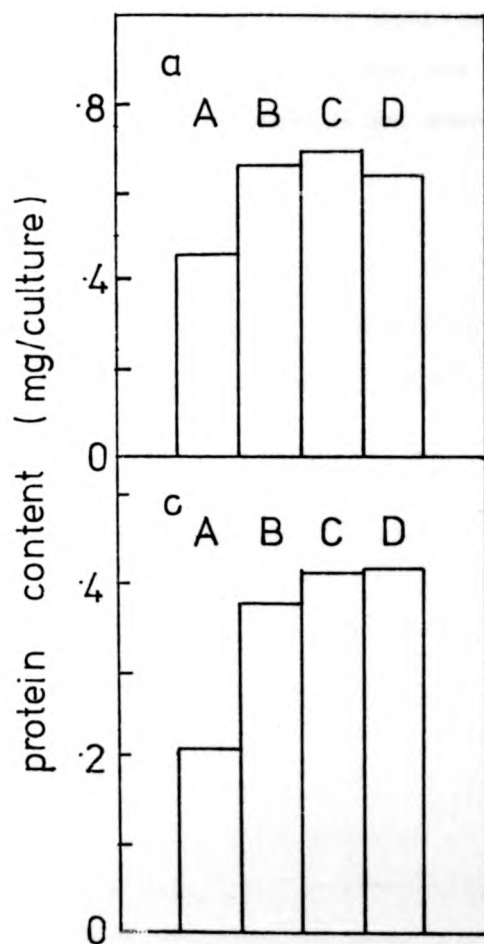
(c) seeded at  $4 \times 10^5$  cells/culture, not confluent

(d) seeded at  $2 \times 10^5$  cells/culture, not confluent

In each case the protein content after four different treatments is shown. A, interferon and poly rI.rC; B, interferon; C, "mock-interferon" and poly rI.rC; and D, "mock-interferon". Note the different scales.

protein content (mg/culture)





value, whether calculated relative to all the controls (Fig. 24b), or just the "interferon only" cultures.

It is clear that there is no all-or-none dependence of cytotoxicity on the stage of the cell cycle at which dsRNA is added.

These experiments would seem to eliminate the most straightforward explanations for resistance, but cannot rule out a dependence on some combination of genotype and phenotype.



FIG.24. Cytotoxic effect in synchronised cells

25 mm vials were seeded with  $1 \times 10^5$  cells/culture and left to grow for 24 hr. The medium was then replaced with GMEM/10% FBS containing 2.5 mM thymidine (Lee and Rozee 1970) for 20 hr. The fluids were removed and the cultures washed with GMEM before adding GMEM/10% FBS containing interferon or "mock-interferon". After 10 hr, the second thymidine block was applied, with interferon or "mock-interferon" again present in the medium.

After 18 hr the fluids were removed, the cultures washed, and fed with GMEM/10% FBS containing interferon or "mock-interferon". As required, cultures were drained, and either used to determine DNA synthesis, or washed free of serum and treated with poly rI.rC for 3.5 hr before washing and estimating protein content. Cultures were also stained to estimate mitotic index (section 2.2.9).

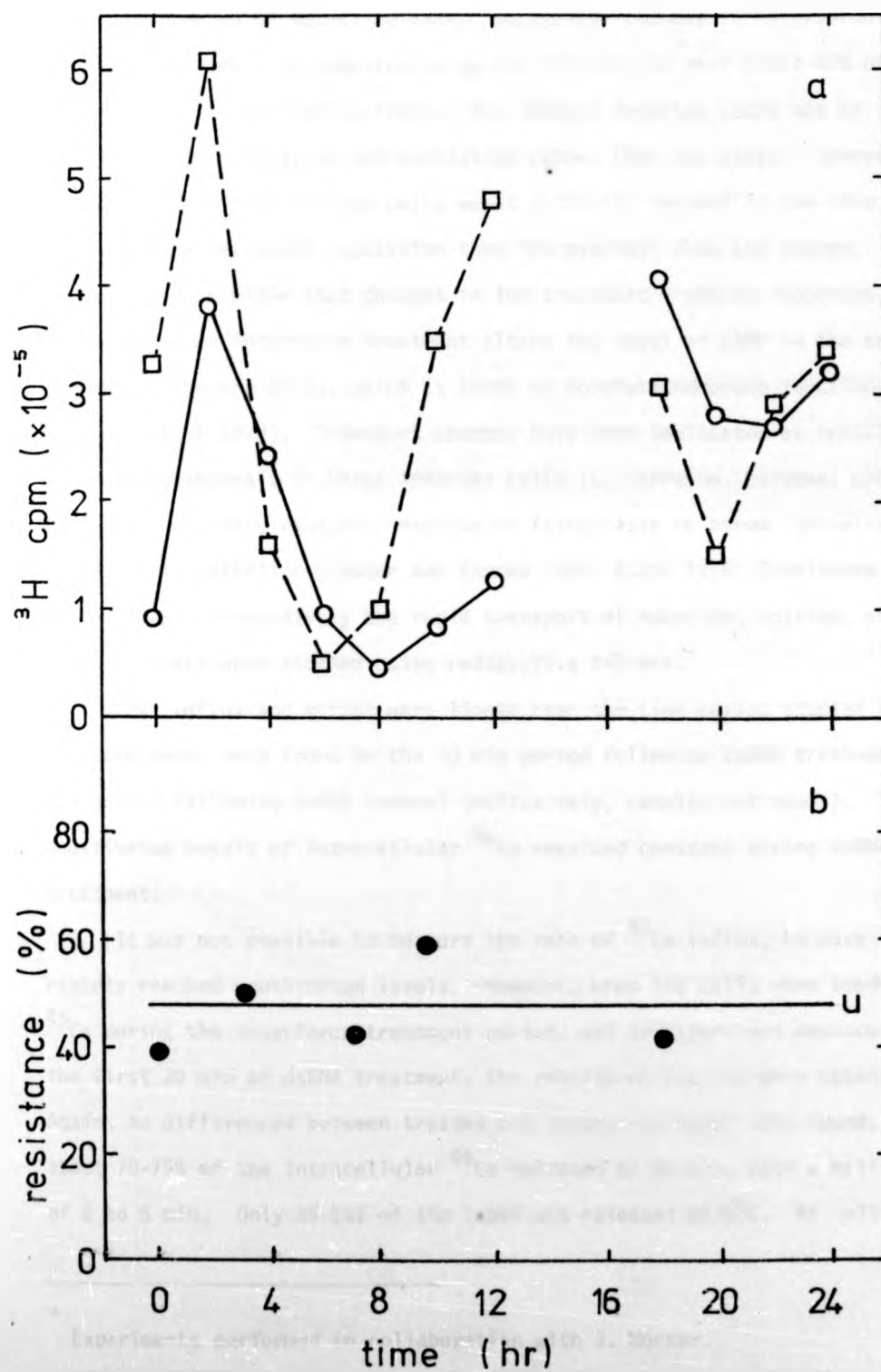
- (a) TCA insoluble  $^3\text{H}$  cpm per culture after 15 min with 5  $\mu\text{Ci/ml}$   $^3\text{H}$  thymidine. No samples were taken between 12 and 18 hr.

- interferon treated
- "mock-interferon" treated

- (b) % resistance to interferon and poly rI.rC, expressed as the ratio of mean protein content of duplicate treated cultures, and the mean protein content of duplicate cultures treated with interferon alone, "mock-interferon" and poly rI.rC, or "mock-interferon" alone. Each point shows % resistance plotted against time of addition of poly rI.rC.

u = unsynchronised cells seeded at the same time as the synchronised cells.

$^3\text{H}$  cpm ( $\times 10^{-5}$ )



\* 5.6. SEARCH FOR SPECIFIC CHANGES EARLY IN THE CYTOTOXIC EFFECT

The problem of detecting early biochemical changes in interferon and dsRNA treated cells is complicated by the observation that about 60% of the cells resist the toxic effect. Any changes detected could not be assigned unambiguously to one population rather than the other. However, it was possible that all the cells would initially respond in the same way, with the resistant population then "recovering" from the change.

It was possible that changes in ion transport might be important, particularly as interferon treatment alters the level of cAMP in the cell (Weber and Stewart 1975), which is known to modulate membrane functions (Korinek et al 1973). Transport changes have been implicated as inhibitors of protein synthesis in virus infected cells (L. Carrasco, personal communication 1976), and in the pleiotypic response of fibroblasts to serum concentration and contact inhibition (Foster and Pardee 1969; Rubin 1975; Cunningham and Pardee 1976). Accordingly the rapid transport of rubidium, calcium, and orthophosphate were studied using radioactive tracers.

$^{86}\text{Rb}$  influx and efflux were linear over the time period studied (Fig.25). No differences were found in the 30 min period following dsRNA treatment or the period following dsRNA removal (efflux only, results not shown). The equilibrium levels of intracellular  $^{86}\text{Rb}$  remained constant during dsRNA treatment.

It was not possible to measure the rate of  $^{45}\text{Ca}$  influx, because it rapidly reached equilibrium levels. However, when the cells were loaded with  $^{45}\text{Ca}$  during the interferon treatment period, and transport out measured during the first 30 min of dsRNA treatment, the results of Fig. 26 were obtained. Again, no differences between treated and control cultures were found, with about 70-75% of the intracellular  $^{45}\text{Ca}$  released by 30 min, with a half-time of 2 to 5 min. Only 15-25% of the label was released at  $0^{\circ}\text{C}$ . At all times

\*

Experiments performed in collaboration with J. Morser.

FIG.25. Rubidium transport kinetics

(a) Rubidium uptake. Vial cultures were treated with interferon or "mock-interferon" as usual. After washing to remove serum, GMEM(Hepes) containing 0 or 50  $\mu\text{g/ml}$  poly rI.rC and 43  $\mu\text{Ci/ml}$   $^{86}\text{RbCl}$  was added. As required, the cultures were transferred to melting ice and rapidly washed three times with cold PBS, before extracting with cold 10% TCA. An aliquot of the acid soluble material was counted and the cpm corrected to cpm per culture (about  $1.6 \times 10^6$  cells). The intracellular Rb content was calculated from the specific activity of the isotope. Each point is the mean of duplicate cultures.

- interferon and poly rI.rC
- interferon
- "mock-interferon" and poly rI.rC
- "mock-interferon"

(b) Rubidium efflux. Vial cultures were treated with interferon or "mock-interferon" in GMEM/2% CS containing 10.8  $\mu\text{Ci/ml}$   $^{86}\text{RbCl}$  for 18 hr and then transferred to ice. The vials were washed free of serum and isotope, warmed rapidly to  $37^\circ\text{C}$  and treated with 0.5 ml of GMEM(Hepes) containing 0 or 50  $\mu\text{g/ml}$  poly rI.rC. At various times the fluids were removed and later sampled for scintillation counting. The cpm have been corrected for sample size to cpm per culture (about  $1.6 \times 10^6$  cells), or as a percentage of the TCA soluble cpm initially present in the cultures (equivalent to about 2.9 nmoles Rb/culture).

Each point is the mean of duplicate cultures

- interferon and poly rI.rC
- interferon alone

"Mock-interferon" treated cultures gave similar results.

time (min)

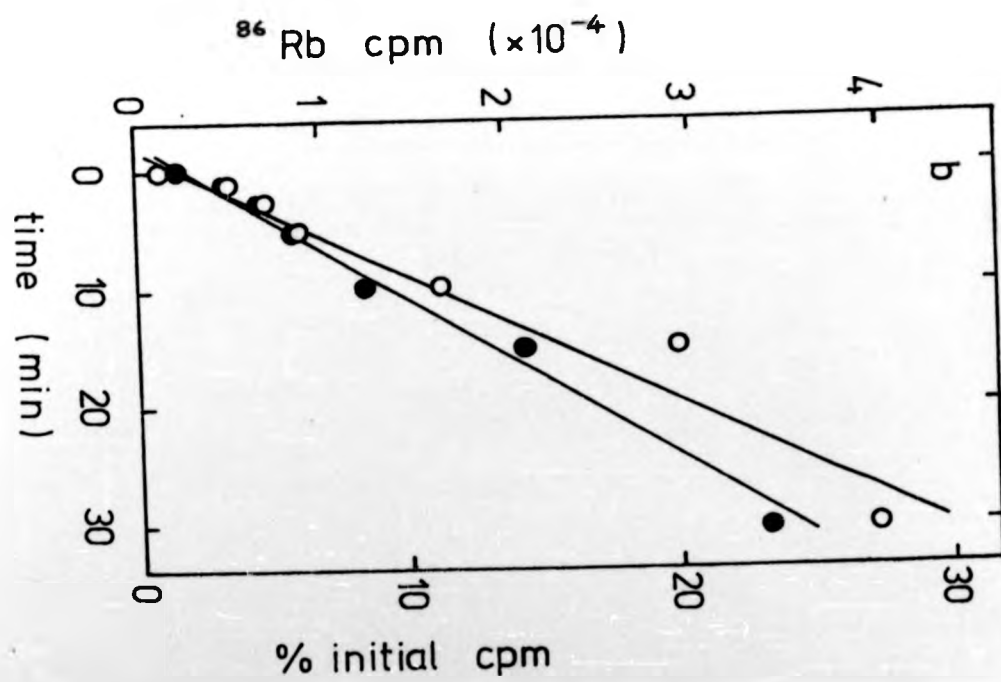
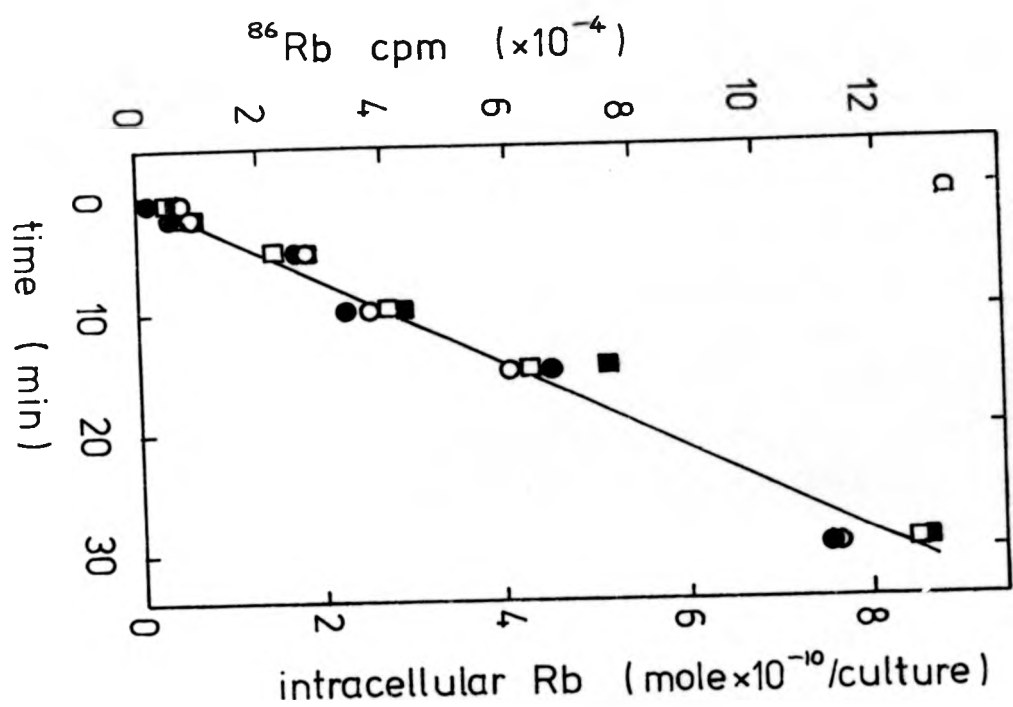


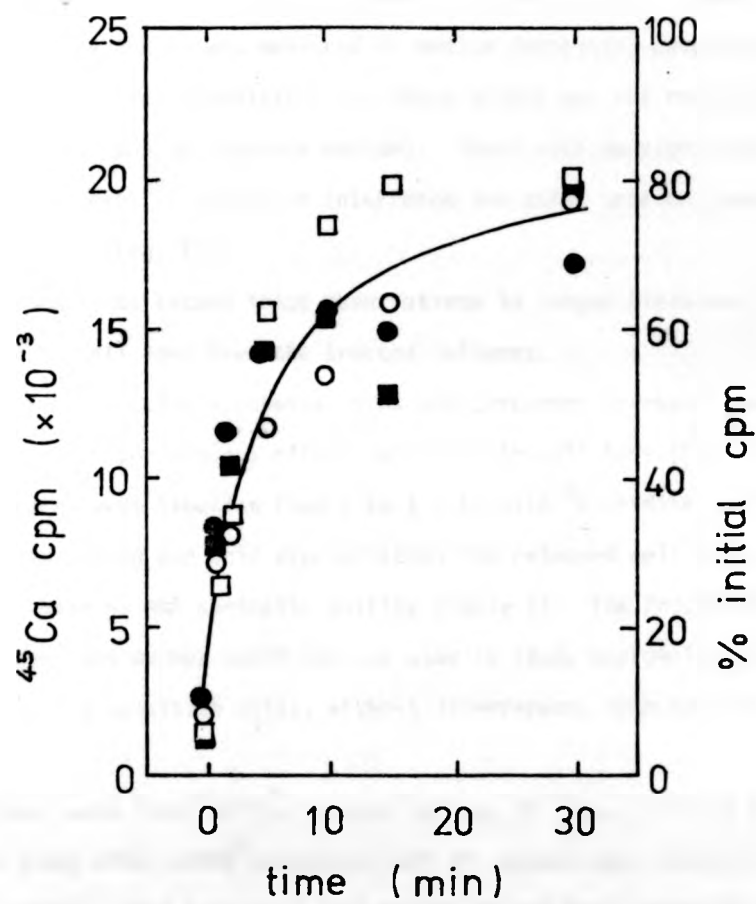


FIG.26. Calcium transport kinetics.

Calcium efflux was measured as described for rubidium (legend to Fig.25b) except that the medium contained  $5.5 \mu\text{Ci/ml } ^{45}\text{CaCl}_2$ . The cpm in the extracellular fluids have been converted to cpm per culture (about  $1.6 \times 10^6$  cells), or as a percentage of the total cpm initially present in the cultures (equivalent to about 5 nmoles Ca. per culture). Each point is the mean of duplicate cultures.

- interferon and poly rI.rC
- interferon
- "mock-interferon" and poly rI.rC
- "mock-interferon"





about 15-20% of the intracellular label was acid insoluble, presumably bound either to the glass or to macromolecules, but this fraction freely exchanged with acid soluble label. None of these parameters altered with the treatment of the cultures.

Whereas the  $^{45}\text{Ca}$  and  $^{86}\text{Rb}$  experiments were performed in complete medium, phosphate influx was measured in medium containing about 450  $\mu\text{M}$  phosphate. Under these conditions the toxic effect was not reduced (59% in low phosphate, 60% in complete medium). There were no significant differences between  $^{32}\text{P}$  influx in interferon and dsRNA treated, and in control cultures (Fig. 27).

Any attempts to extend these observations to longer times would be complicated by cell loss from the treated cultures.

When macromolecular synthesis rates and precursor transport were studied, the most conspicuous effects were due to cell loss (Fig. 17, 20). If the cultures were labelled from 3 to 3.5 hr with  $^3\text{H}$  uridine, and the 3.5 hr washes pooled and acid precipitated, the released cell debris could be shown to have no RNA synthetic ability (Table 7). The fractionation into monolayer and washes could thus be used to study macromolecular synthesis in the sensitive cells, without interference from the resistant cells.

Cultures were labelled for various periods of time, starting at different times after dsRNA treatment, but all pulses were stopped at 3.5 hr, when the cultures were separated into monolayer and wash fractions, and acid insoluble incorporation determined. Using this protocol, any disturbance of the cells between 1 and 3.5 hr was avoided, so all the sensitive cells should be present, as debris, in the wash fraction.

Fig. 28 shows the results when  $^3\text{H}$  uridine was used. The cellular protein was previously labelled with  $^{35}\text{S}$  methionine so that the protein content of the wash fraction could be determined. In this experiment, 37% of the protein was recovered in the 3.5 hr washes, and less than one-tenth

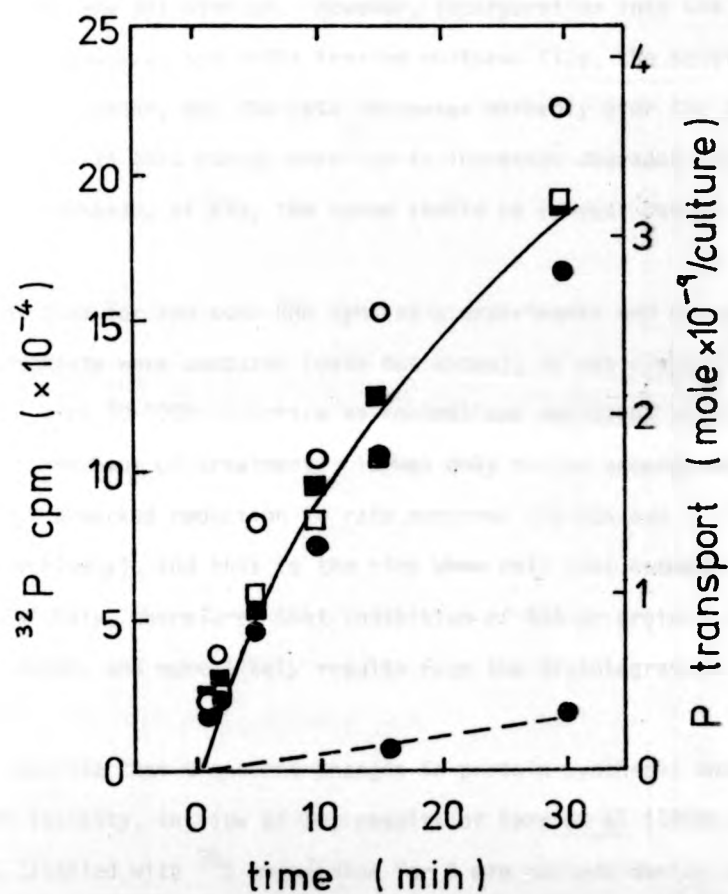
FIG.27. Orthophosphate transport kinetics

Phosphate uptake was measured as described for rubidium (legend to Fig.25a) except that GMEM-P was used so the medium contained 30  $\mu\text{Ci/ml}$   $^{32}\text{P}$  orthophosphate and 450  $\mu\text{M}$  total orthophosphate. At various times TCA soluble and insoluble cpm per culture were determined, and converted to moles orthophosphate per culture (about  $1.6 \times 10^6$  cells).

Results are means of duplicate cultures.

- interferon and poly rI.rC
- interferon
- "mock-interferon" and poly rI.rC
- "mock-interferon"

The broken line shows the incorporation into acid insoluble material, which was approximately the same for each of the four treatments.



of this was removed with the dsRNA at 1 hr. The results (Fig. 28a) show that RNA synthesis was approximately linear in the control cultures, and also in the monolayer fraction of the interferon and dsRNA treated cultures (i.e. the resistant population). The rates expressed relative to the amount of protein present are all similar. However, incorporation into the wash fraction of the interferon and dsRNA treated cultures (i.e. the sensitive population) is not linear, but the rate decreases markedly over the 3.5 hr period (Fig. 28b). If this change were due to increased degradation, rather than decreased synthesis, of RNA, the curve should be convex, rather than concave.

When the rates for two such RNA synthesis experiments and two protein synthesis experiments were compared (data not shown), it was clear that the sensitive cells were 50-100% as active as control and resistant cells, at least for the first hour of treatment. It was only in the second and third hours that a marked reduction in rate occurred (10-50% and 2-15% of controls, respectively), and this is the time when cell loss commences. It seems highly unlikely, therefore, that inhibition of RNA or protein synthesis precedes cell death, and more likely results from the disintegration of the cell structure.

It was possible that transient changes in protein synthesis would correlate with toxicity, in view of the results of Kerr *et al* (1974b). When cultures were labelled with  $^{35}\text{S}$  methionine for 5 min periods during the first hour of dsRNA treatment, there were no differences between interferon-treated and control cultures (data not shown).

Attempts were also made to detect qualitative changes in macromolecular synthesis. When cultures, labelled with  $^3\text{H}$  uridine 0 to 0.75 hr or 3 to 3.75 hr after dsRNA treatment, were extracted with phenol and chloroform and the RNA sedimented on sucrose gradients, profiles showing peaks of 4-5S, 18S and 28S material superimposed on a background of heterogeneous, unprocessed, material were obtained. The profiles were identical for treated and control cultures (data not shown).

FIG.28. RNA synthesis during the cytotoxic effect.

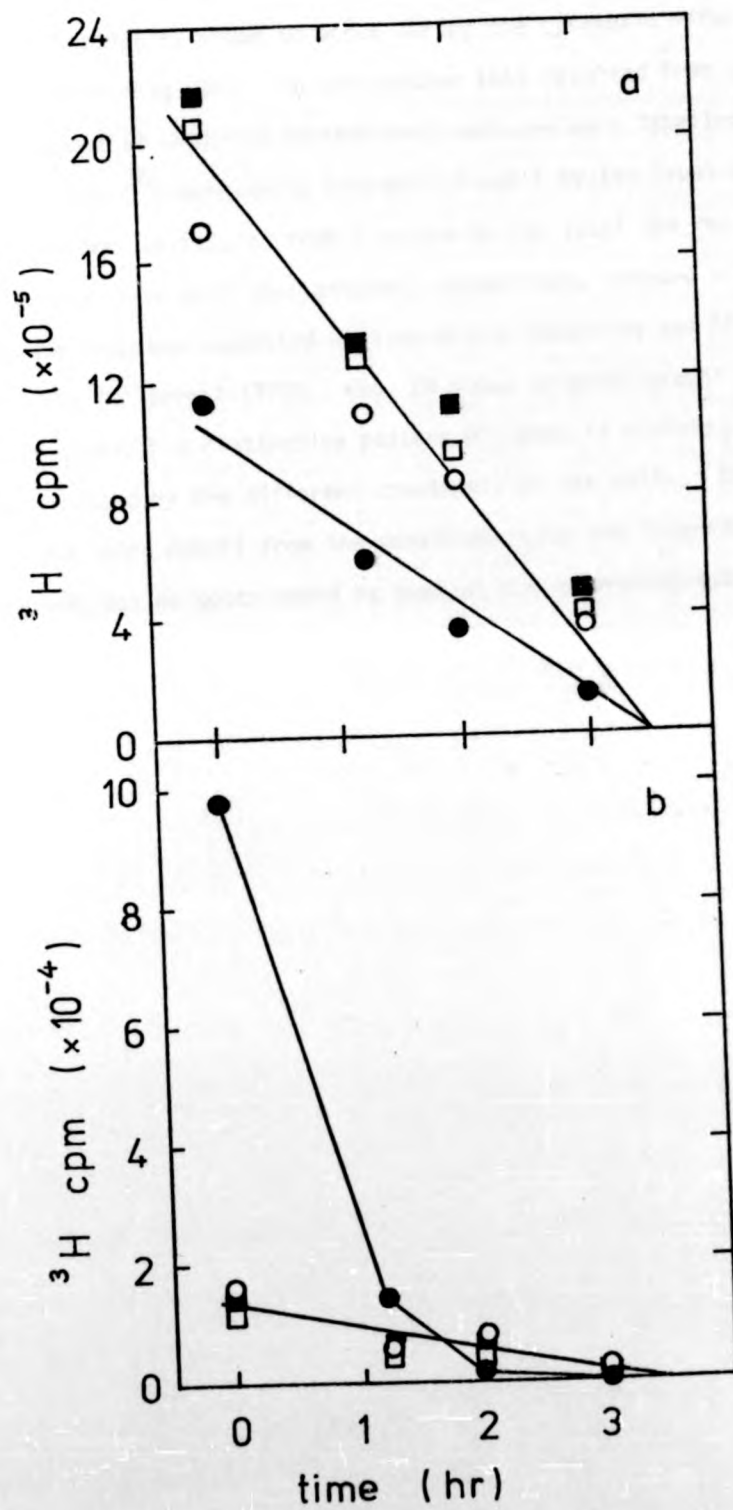
vial cultures were treated with interferon and poly rI.rC as usual. At various times,  $^3\text{H}$  uridine was added to the medium to a final concentration of  $10\text{ }\mu\text{Ci/ml}$ , and was left on the cells until 3.5 hr, when all the cultures were washed three times with PBS. The washes were pooled, and the washes and the monolayer were washed three times with TCA and once with ethanol. The dried precipitates were solubilised and counted.

Parallel cultures, which had been labelled beforehand with  $^{35}\text{S}$  methionine ( $2.5\text{ }\mu\text{Ci/ml}$  in GMEM-M for 30 min at  $37^\circ\text{C}$ ), were also treated with poly rI.rC but not with  $^3\text{H}$  uridine. The resulting monolayers were solubilised in alkali and used for Lowry protein estimation. The  $^{35}\text{S}$  radioactivity, which was precipitated with TCA from the wash fractions, was used to estimate the protein content of the wash fractions relative to the monolayer fractions.

In the Figure, acid insoluble  $^3\text{H}$  cpm per culture is plotted against the time of the start of the  $^3\text{H}$  uridine labelling period for ●, interferon and poly rI.rC; ○, interferon; ■, "mock-interferon" and poly rI.rC; and □, "mock-interferon" treated cultures. Each point is the mean of triplicate cultures.

(a) Monolayer fractions. Protein contents (Lowry) were 253, 339, 410, and  $443\text{ }\mu\text{g/culture}$  respectively.

(b) Wash fractions. Protein contents (from  $^{35}\text{S}$  cpm) were 145, 51, 52, and  $34\text{ }\mu\text{g/culture}$  respectively.





One specific change is known to occur during the cytotoxic effect - interferon is produced (Fig. 22). To see whether this resulted from gross changes in the pattern of proteins synthesised, cultures were labelled with high specific activity  $^{35}\text{S}$  methionine between 0.5 and 1 hr (to label both sensitive and resistant cells), or from 3 to 3.5 hr (to label the resistant cells). The cultures were acid precipitated, solubilised, treated with nucleases, and the proteins separated by isoelectric focussing and SDS polyacrylamide gels (O'Farrell 1975). Fig. 29 shows autoradiographs of the dried gels. Although a distinctive pattern of spots is visible, there were no changes induced by the different treatments of the cells. The 3.5 hr wash samples (containing debris from the sensitive cells and interferon) were also analysed, but no spots could be seen on the autoradiographs.



FIG.29. The pattern of proteins synthesised early in the cytotoxic effect. \*

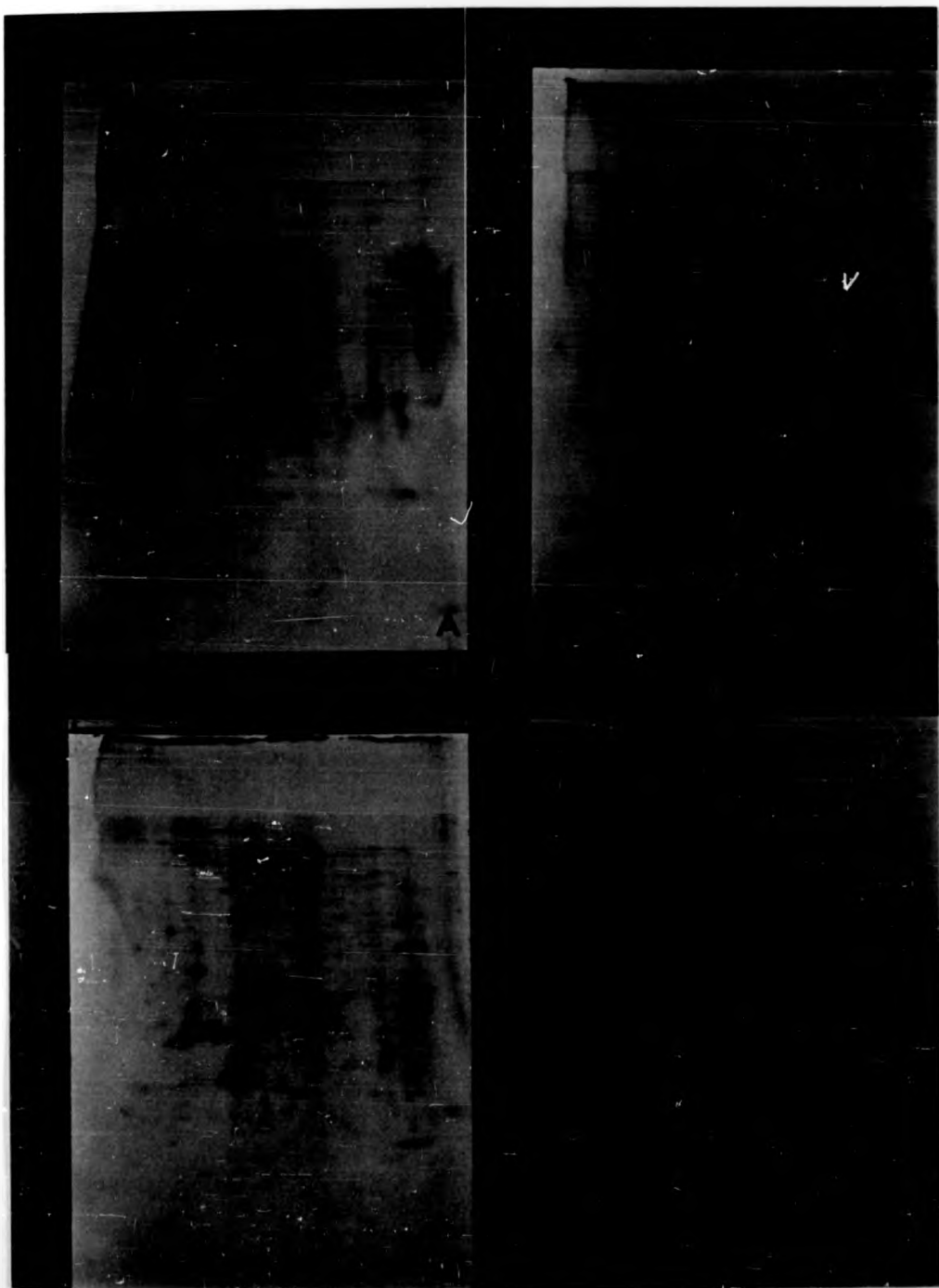
25 mm vial cultures of cells were treated with 200 u/ml interferon and 50 µg/ml poly rI.rC, as usual. 30 min after poly rI.rC treatment the cultures were washed with GMEM-M, drained, and 0.5 ml GMEM-M, containing 250 µCi <sup>35</sup>S methionine, added. After a further 30 min at 37°C the cultures were washed three times each with PBS, 10% TCA, ethanol, and ether, and allowed to dry.

Analysis of labelled proteins was basically according to O'Farrell (1975). Each monolayer was scraped into 100 µl of solution B, sonicated, treated with DNase (buffer C), and then with solution A and solid urea. The main differences from O'Farrell's procedure were that SDS was also added to 0.1% before applying to the isoelectric focussing gels, and these were run with the acid in the top reservoir (to minimise interference from oligonucleotides). The isoelectric focussing gels were equilibrated with SDS before laying across a 7.5-30% gradient polyacrylamide slab with a stacker (section 2.6.2). After electrophoresis the slabs were stained and dried down (section 2.6.3) for autoradiography.

5 day autoradiographs for A, interferon and poly rI.rC; B, interferon; C, "mock-interferon" and poly rI.rC; and D, "mock-interferon" treated cultures are shown. The pH gradient was from a parallel isoelectric focussing gel. The molecular weight markers were: 165 K daltons, thyroglobulin; 95 K, phosphorylase A; 77 K, transferrin; 68 K, BSA; 45 K, ovalbumin; 34 K, carbonic anhydrase; 15.5 K, myoglobin. The antiviral effect of L929 cell interferon ran as 3 peaks on isoelectric focussing gels with isoelectric points of 5.60, 6.45, and 7.1, and as 2 peaks on SDS gradient gels with apparent molecular weights of 32 K and 25 K daltons (data not shown).

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\* Experiments performed by J. Morser.



### 5.7. DISCUSSION

When either LA9 or L929 cells are treated with interferon and dsRNA there is rapid and extensive cell death. However, in both cell types, a significant proportion (50-70%) of the cells remain attached to the substratum and continue macromolecular synthesis. These cells remain viable for at least 24 hr, whereas the sensitive cells are released into the medium as subcellular debris. The proportion of sensitive cells cannot be increased by increasing the dose of interferon or dsRNA, or by repeated administration of dsRNA, and is independent of the length of interferon treatment.

Stewart *et al* (1972a) have described the cytotoxic effect as involving total destruction of the cell monolayer. However, this corresponds to 8% trypan blue excluding cells and 29% residual neutral red stain incorporation. In Fig. 15 I compare neutral red stain incorporation with protein estimations and find a similar proportion (37%) of residual stain incorporation. It would seem, therefore, that there may be a resistant population in all L cell stocks. The precise proportion depends upon the assay used, and may be minimised by counting trypan blue excluding cells after trypsinisation, and maximised by the protein assay described here.

Recognising that cells may be sensitive or resistant to the toxic effect raises several interesting questions.

Firstly, what decides whether a particular cell is sensitive or resistant? The results presented here rule out simple explanations based on stable inheritance of resistance, cell density, or on stage of the cell cycle (Table 8, Figs. 23,24). However, the effect is not sufficiently reproducible to exclude small variations with any of these parameters, and a combination of phenotypic and genotypic factors could be important.

It is not known whether the resistant/sensitive decision is made before interferon treatment, during interferon treatment, or during dsRNA treatment. An interferon-treated cell could adopt one of two states; one

in which it will be sensitive to dsRNA, or one in which it will be resistant. On the other hand, the decision may only be made after dsRNA treatment. These alternatives cannot be distinguished until some method of separating the two populations before cell death can be found.

Secondly, do the sensitive or the resistant cells produce interferon? A variety of different methods for superinducing the interferon response (Tan *et al* 1970) involve procedures which cause extensive cytopathic effects. The data in Fig. 22 do not discriminate between production by the sensitive or by the resistant cells, because the bulk of synthesis occur during the period of cell death. Unfortunately, without a way of separating the two populations before dsRNA treatment, it is not possible to distinguish the alternatives.

Thirdly, the question of what triggers the toxic effect remains unanswered. Stewart and his colleagues have described the ultra-structural changes as involving alterations in nuclei, mitochondria and microfilaments (Heremans *et al* 1976). However, at this time, signs of lysosomal activation are evident (W. E. Stewart II., personal communication 1975), and the changes are probably the result, rather than the cause, of toxicity. The initial interaction with dsRNA must be at the membrane, and a membrane trigger for toxicity cannot be excluded, particularly in view of the changes in cAMP levels during interferon treatment (Weber and Stewart 1975). We were unable to show any changes in ion or precursor transport (Figs. 25, 26, 27, 17, 20) as might have been expected if action was directly at the membrane. No significant differences in macromolecular synthesis, quantitative or qualitative, were seen (Figs. 17, 20, 28, 29). However, as long as the toxic effect is only partial the sensitivity of the experiments is limited, and even if changes could be detected, their relevance in a causative chain of events would need to be established.

SECTION 6  
PREPARATION AND CHARACTERISATION OF A  
CELL-FREE PROTEIN SYNTHESISING SYSTEM  
FROM MONOLAYER GROWN L929 CELLS

## 6. PREPARATION AND CHARACTERISATION OF A CELL-FREE PROTEIN SYNTHESISING SYSTEM FROM MONOLAYER GROWN L929 CELLS.

### 6.1. INTRODUCTION

L929 cells can be selected to grow either in monolayers or in suspension. Whereas the suspension cells are convenient for experiments where large numbers of cells are required, and for making cell-free protein synthesising systems, the monolayer grown cells are convenient for experiments involving repeated media changes, such as investigating virus growth or interferon action. It was desirable to obtain a cell-free protein synthesising system from monolayer cells, so that biochemical changes could be studied in parallel in vivo and in vitro. The most important characteristic of the system was that it should reflect the metabolic state of the cells, rather than for it to translate added mRNAs with high efficiency.

Standard methods for preparing cell-free systems have been described (Eggen and Shatkin 1972). Basically, the cells are collected, washed homogenised in a small volume of hypotonic buffer, made to contain suitable concentrations of potassium and magnesium ions, and a supernatant fraction prepared at 10000 or 30000 g. This procedure was not applicable to the monolayer L929 cells in use in our laboratory. Firstly, the rapid cooling essential for an active system (Kerr et al 1966) is technically difficult for monolayers of cells on glass bottles, particularly when large numbers of cells are required. Secondly, and most important, these cells were very resistant to most mechanical breakage methods tried. Even after swelling the cells in hypotonic buffer, dounce and Potter homogenisers only produced low percentage breakage, especially at the high cell densities required. It was necessary to use sonication to produce an effective elongating system, but this system was defective in initiation (results not shown).

However, non-ionic detergents have been used successfully to rupture cells. Austin and Kay (1975) lysed Hela cells using the non-ionic detergent Nonidet P-40, and Ball and White (1976) have described a long-lived coupled

transcription and translation system in which the non-ionic detergent Triton N101 is present. It was thus possible that the presence of non-ionic detergents would not inhibit protein synthesis.

In addition, the advantages of breaking the cells in isotonic conditions have been pointed out (Villa-Komaroff *et al* 1975). Hela cell S-30s made by douncing in isotonic buffer were at least 10 times more active in elongation and initiation than extracts made after swelling in hypotonic buffer.

Finally, the description of an active nuclear RNA synthesising system, prepared using detergent to lyse cells whilst still attached to the petri dish (Tsai and Green 1973), led me to develop the system described below, in which cells are rapidly cooled on the dish, lysed in isotonic buffer using a non-ionic detergent, and the cytoplasmic fraction is collected and assayed without centrifugation.

## 6.2. PROCEDURE FOR PREPARING THE CELL-FREE SYSTEM

Phase-contrast microscopy suggested that 0.1% V/V Triton N101 (polyoxyethyleneglycol (9-10) nonylphenol) in isotonic buffer was adequate for lysing L929 cells. It was observed that 50 $\mu$ l of detergent would spread to cover most of a 50mm plastic petri dish in about 2 min, lysis being virtually instantaneous. Higher concentrations of detergent spread more rapidly but were thought to be more likely to inhibit protein synthesis, whereas low concentrations spread slowly and did not cause complete lysis. The nuclei remained intact and attached to the dish even when 1% V/V Triton N101 was used. Triton X-100 and Nonidet P40 produced slower lysis and were not investigated further.

The procedure adopted (see section 2.3.5) was to grow the cells to confluence in 140 mm dishes, cool rapidly by transferring to melting ice, and then to wash three times, rapidly, with cold PBS before draining and washing with the extraction buffer. This was removed, and the dish floated horizontally on ice-cold water and five 100 $\mu$ l aliquots of 0.1% V/V Triton N101 in extraction buffer applied. After 3 min for the cells to lyse, the dish was tipped and the cytoplasmic fraction collected over the next 2 min. This was either assayed directly or stored in liquid nitrogen.

The average volume of cytoplasm collected was 650-700 $\mu$ l per dish, with a protein concentration of  $2.39 \pm 0.79$  mg/ml (mean and standard deviation, 29 extracts). The yield of protein depended on the cell density, but within a series of dishes seeded at the same density, the concentration of protein was relatively constant. The yield of protein (about 30-40 $\mu$ g/10<sup>6</sup> cells) was only about 10% of the total cellular protein, so presumably most of the cellular protein remained associated with the nucleus or cytoskeleton. The protein concentration is probably equivalent to conventional lysis procedures using homogenisation in 10 packed-cell volumes of buffer.



FIG.30. Characteristics of endogenous protein synthesis by detergent extracts.

Extracts of L929 cells were prepared (section 2.3.5) and assayed immediately (section 2.3.6).

(a) Kinetics of protein synthesis. 100  $\mu$ l incubations contained:

○ , 4  $\mu$ Ci of  $^{35}\text{S}$  methionine, or □ , 25  $\mu$ Ci of  $^3\text{H}$  leucine, and unlabelled amino acids, energy supply, and 127  $\mu$ g extract protein. 10  $\mu$ l aliquots were taken as required for acid precipitation.

(b) Dependence on concentration of extracts. 50  $\mu$ l incubations contained 10  $\mu$ Ci of  $^{35}\text{S}$  methionine, unlabelled amino acids and energy supply, and various concentrations of extract protein, diluted with 0.1% V/V Triton N101 in extraction buffer. After 30 min at 30°C, two 10  $\mu$ l aliquots were taken for acid precipitation, and the mean incorporation found.

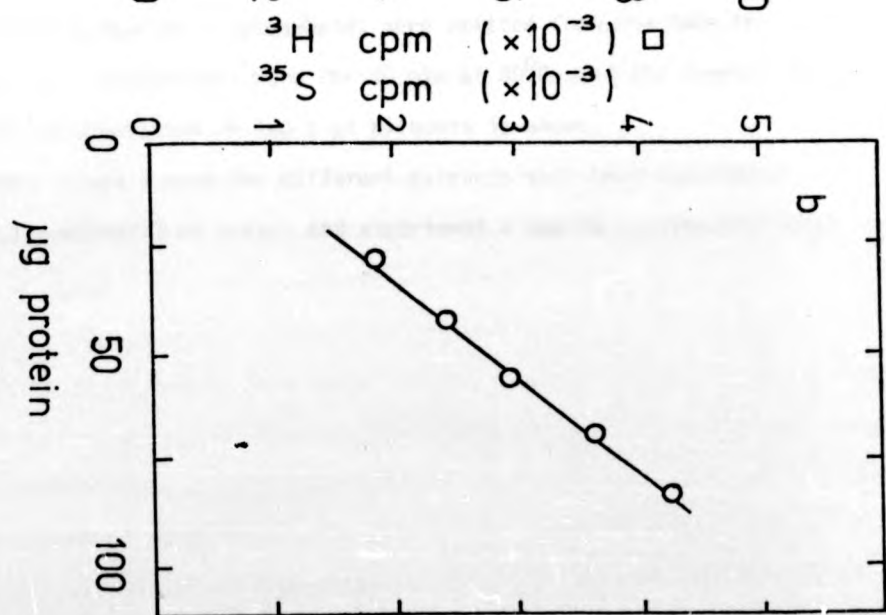
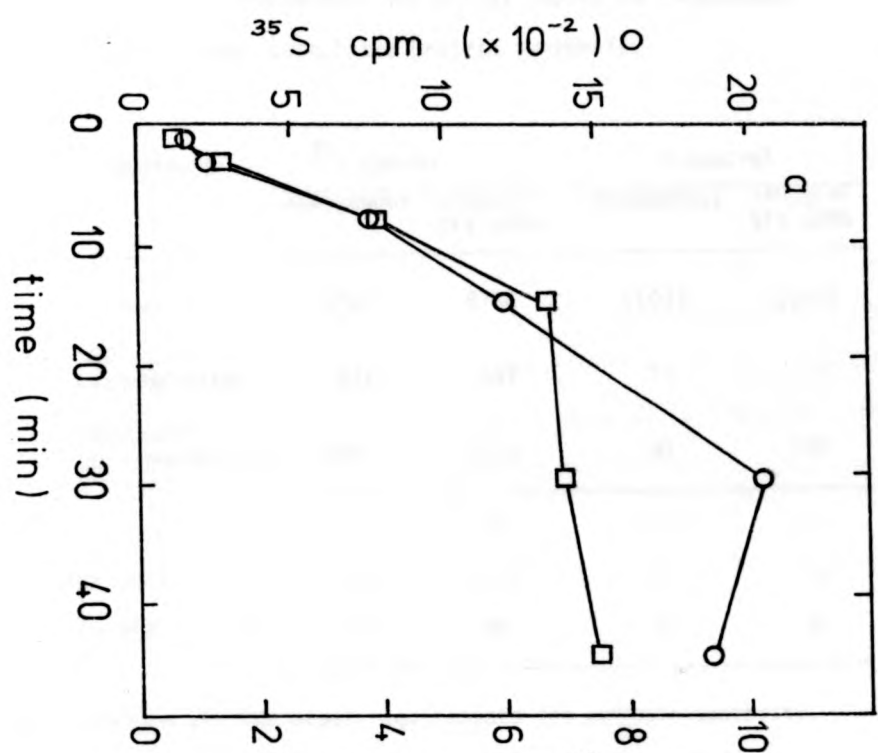


TABLE 9. Effects of inhibitors and energy supply on endogenous and SFV mRNA stimulated protein synthesis.

Experiment No.	Additions	<sup>35</sup> S cpm/5μl		% control	
		endogenous	+40μg/ml SFV mRNA	endogenous	+40μg/ml SFV mRNA
1	none	4360	5766	(100)	(100)
	100 μg/ml cycloheximide	477	392	11	7
	100 μg/ml chloramphenicol	3867	5769	87	100
2	none	2097	3453	(100)	(100)
	10 mM NaF	730	1114	35	32
	no ATP,GTP,CTP	659	ND	31	ND

Incubations contained all the usual requirements for protein synthesis, except that nucleoside triphosphates were omitted from one tube in experiment 2. Incubations were for 30 min at 30°C, and the average acid insoluble incorporation in two 5 μl aliquots is shown.

Experiments 1 and 2 used two different extracts such that experiment 1 had 59 μg protein/50 μl assay, and experiment 2 had 65 μg protein/ 50 μl assay.

### 6.3 CHARACTERISTICS OF PROTEIN SYNTHESIS

When these cytoplasmic extracts were incubated under conditions allowing protein synthesis, incorporation of  $^{35}\text{S}$  methionine or  $^3\text{H}$  leucine into hot acid insoluble material took place with the kinetics illustrated (Fig. 30a). After a short lag of 1-2 min, synthesis proceeded more or less linearly until 20-30 min when a plateau was reached. Occasionally a small reduction in incorporation occurred after continued incubation.

Table 9 shows the effects of some inhibitors on protein synthesis. There was only slight inhibition when mitochondrial protein synthesis was prevented by chloramphenicol, whereas cycloheximide was fully inhibitory. This suggests that synthesis is occurring on cytoplasmic ribosomes. Synthesis was increased by added mRNA and was dependant on an energy supply.

The rate of endogenous protein synthesis is proportional to the amount of extract protein used (Fig. 30). This experiment was performed keeping the ionic conditions and the concentration of detergent constant, and the magnesium dependence curve was shown to be similar at two different protein concentrations (data not shown). Because of the large endogenous pool of  $^{32}\text{S}$  methionine in the extracts (Fig. 32), the dependence of the absolute rate of protein synthesis on protein concentration is not linear (Fig. 30), but a concave curve.

Both endogenous and SFV mRNA-stimulated protein synthesis rates were shown to be insensitive to the addition of extra detergent, up to an extra 0.1% final concentration (data not shown).

Fig. 31 shows an experiment in which the optimal ionic conditions for making the extracts were investigated. A series of dishes of cells were lysed in buffers of different ionic composition, and assayed for protein concentration, and for incorporation of  $^{35}\text{S}$  methionine in the absence and presence of added mRNA. The graphs show several features. Firstly, the relative rate of protein synthesis is highly reproducible from one dish of cells to the next. Secondly, these curves are similar to ones obtained by

FIG.31. Effect of ionic conditions on activity of detergent extract

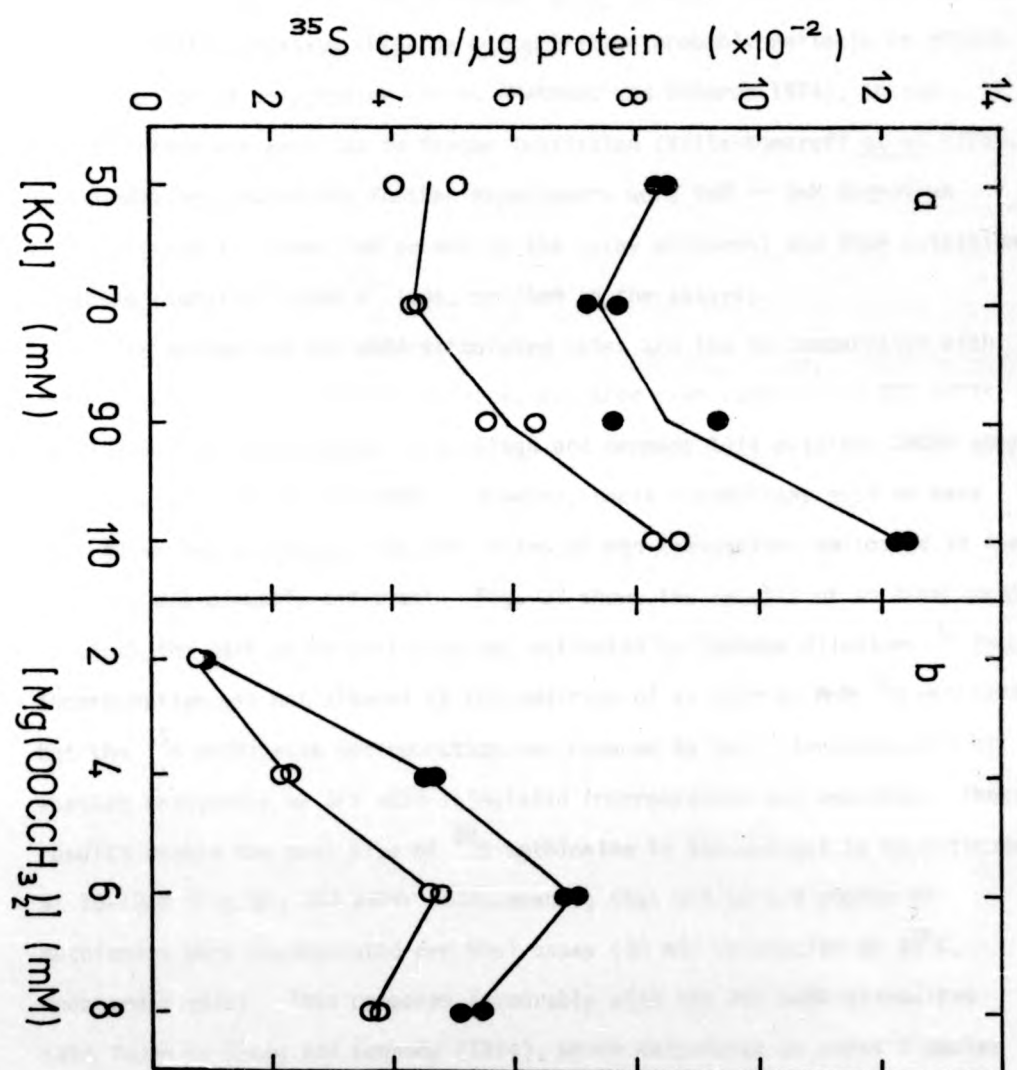
Duplicate 140 mm petri dish cultures of L929 cells were lysed in buffers of different ionic composition and assayed directly in 50  $\mu$ l incubations containing 9  $\mu$ Ci  $^{35}$ S methionine, 0 or 2  $\mu$ g SFV mRNA, 35  $\mu$ l of extract, and other components for protein synthesis. After 30 min at 30°C duplicate 10  $\mu$ l aliquots were acid precipitated. Each pair of points shows the  $^{35}$ S incorporation per  $\mu$ g extract protein for extracts from two separate petri dishes of cells.

(a) lysed in 0.1% V/V Triton N101, 30 mM Hepes KOH, 1mM DTT, 6 mM  $\text{Mg}(\text{OOCCH}_3)_2$  and various concentrations of KCl.

(b) lysed in 0.1% V/V Triton N101, 30 mM Hepes KOH, 1 mM DTT, 90 mM KCl and various concentrations of  $\text{Mg}(\text{OOCCH}_3)_2$ .

○ endogenous incorporation

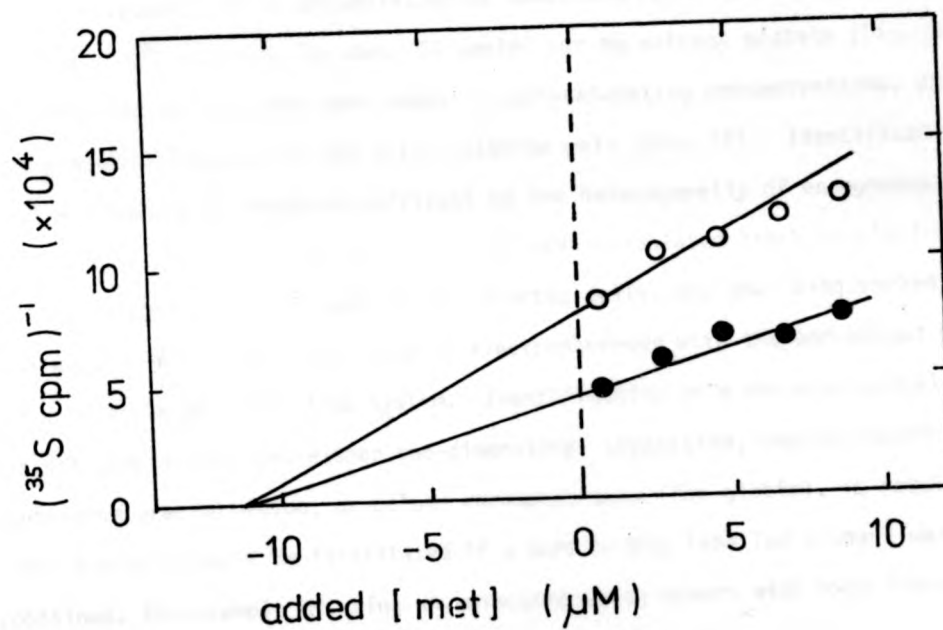
● incorporation in presence of 40  $\mu$ g/ml SFV mRNA



varying the assay, rather than extraction, conditions (data not shown), so there were no major irreversible changes due to making the extract under different conditions. Thirdly, the magnesium optimum, of about 4 mM in the assay, is about the same as that found by other workers using non gel-filtered suspension L929 cell extracts (Friedman *et al* 1972a). The increase in protein synthesis with potassium chloride concentration probably reflects an effect on elongation of polypeptide chains (Mathews and Osborne 1974), so the concentration was kept low to favour initiation (Villa-Komaroff *et al* 1975). The conditions chosen for further experiments were 4mM or 6mM magnesium acetate (that is, about 3mM or 4mM in the assay mixtures) and 90mM potassium chloride (total of 105mM  $K^+$  ions, or 75mM in the assays).

The endogenous and mRNA-stimulated rates are low in comparison with mRNA-stimulated preincubated extracts, prepared from suspension L929 cells by conventional procedures (e.g. Clegg and Kennedy 1974 obtained 38000 cpm/5 $\mu$ l aliquot using SFV 26S mRNA). However, these comparisons must be made on a molar basis, because the pool sizes of non-radioactive aminoacid in the extracts are probably different. Fig. 32 shows the results of an experiment in which the methionine pool size was estimated by isotope dilution.  $^3H$  leucine incorporation was not altered by the addition of as much as 8 $\mu$ M  $^{32}S$  methionine, but the  $^{35}S$  methionine incorporation was reduced by half, independantly of whether endogenous or SFV mRNA-stimulated incorporation was measured. These results enable the pool size of  $^{32}S$  methionine in the extract to be estimated at 10-12 $\mu$ M (Fig.32, and other data), meaning that 0.8 to 0.9 pmoles of methionine were incorporated per 50 $\mu$ l assay (30 min incubation at 30°C, endogenous rate). This compares favourably with the 26S mRNA-stimulated rate found by Clegg and Kennedy (1974), which calculates at about 2 pmoles per 50 $\mu$ l assay (100 min incubation at 30°C; calculation assumes negligible  $^{32}S$  methionine present in the gel-filtered extract).







#### 6.4. EFFECT OF ADDED mRNA

Adding total polyA(+) RNA from the cytoplasm of SFV-infected BHK 21 cells (SFVmRNA) resulted in a stimulation of incorporation of  $^{35}\text{S}$  methionine into hot acid insoluble material (Table 9). A 26S fraction of this mRNA (26S mRNA) was similarly stimulatory, as was the polyA(+)RNA from rabbit reticulocyte polysomes (globin mRNA).

When increasing amounts of 26S mRNA were added to the extract, it was found to saturate at a concentration of about 30mM (molecular weight  $1.6-1.8 \times 10^6$  daltons) or about 25 pmoles per mg extract protein (Fig. 33). 26S mRNA and globin mRNA were added at near-saturating concentrations, and the products analysed on SDS polyacrylamide gels (Fig. 34). Identification of the products is rendered difficult by the heterogeneity of endogenous products, but the band marked on the 26S mRNA-stimulated track co-electrophoresed with SFV capsid protein labelled in infected cells, and the band marked on the globin mRNA-stimulated track co-electrophoresed with the endogenous product of a reticulocyte cell-free system. Identification on a one-dimensional gel is not conclusive, and either two-dimensional separation, peptide mapping, antiserum precipitation, or column chromatography (for globin), is required. This analysis could be facilitated if a more highly labelled product were obtained, for example by using an aminoacid which occurs with high frequency in proteins.

The most important conclusion from Fig. 34 is that discrete products are made by the system, and that the polypeptides directed by added mRNAs resemble the products formed in other systems (see Fig. 1). This shows that the detergent extracts can utilise added mRNA and can initiate correctly, but it is also clear that some of the stimulation is due to increased incorporation into products encoded by the endogenous mRNA.

Two other questions are of interest. Firstly, for how long can the extract continue to initiate new polypeptide chains? Secondly, how much of the stimulation by added mRNA can be explained by competition for ribonucleases?

FIG.33. Dependence of protein synthesis on concentration of mRNA

50  $\mu$ l incubations containing 75  $\mu$ g of extract protein, 10.9  $\mu$ Ci  $^{35}$ S methionine, various concentrations of 26S mRNA, and other components for protein synthesis, were incubated for 30 min at 30°C before taking triplicate 2.5  $\mu$ l aliquots for acid precipitation.

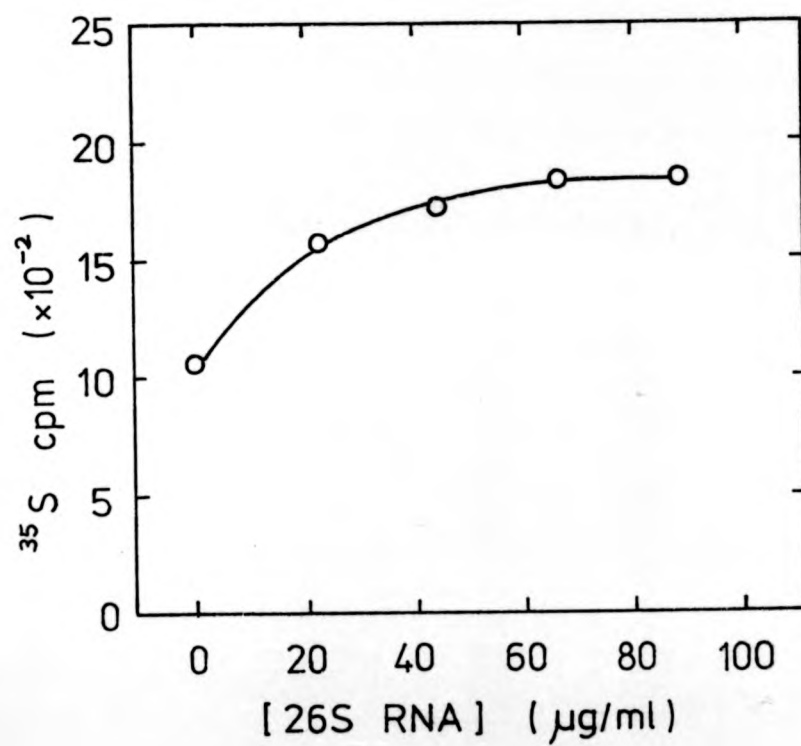


FIG.34 Products of cell-free protein synthesis.

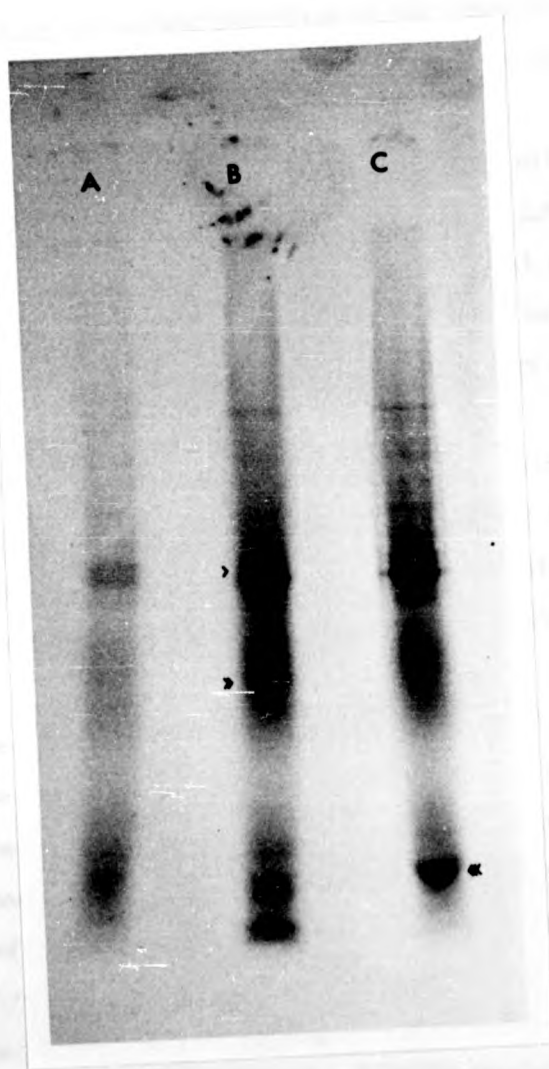
50  $\mu$ l incubations containing 27  $\mu$ Ci  $^{35}$ S methionine, 48  $\mu$ g extract protein, and other components for protein synthesis, were incubated with 2  $\mu$ g of globin or 26S mRNA for 30 min at 30°C. Samples were taken for acid precipitation and the remainder treated with RNase A and prepared for electrophoresis (section 2.6.2). The dialysed incubations were loaded onto 7.5 - 30% gradient gel, and electrophoresed at 14 mA for 18 hr. The gel was impregnated with PPO and fluorographed at -70°C for 4 days (section 2.6.3).

- A, endogenous products, 22000 cpm loaded
- B, 26S mRNA stimulated, 56000 cpm loaded
- C, globin mRNA stimulated, 54000 cpm loaded.

The markers, run on parallel tracks, were SFV envelope (single arrow) and capsid (double arrow) proteins, and rabbit globin (triple arrow).

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### 6.5. INITIATION OF POLYPEPTIDES

The fact that fluoride inhibits protein synthesis in these extracts (Table 9) implies that polypeptide chain initiation is required for continued synthesis (Obrig et al 1971) Edeine is a better characterised inhibitor than fluoride, and has been shown to inhibit initiation at the stage of 60S joining, with excellent discrimination between elongation and initiation (Obrig et al 1971).

Fig. 35a shows the kinetics of protein synthesis with or without SFV mRNA and with various concentrations of edeine. Edeine at 2 $\mu$ M can prevent most of the stimulation due to added mRNA. The residual stimulation could be due to some of the mRNA entering initiation complexes before the edeine inactivates the ribosomes, or due to non-specific effects on incorporation by the added mRNA.

Fig. 35b shows the stimulation due to added mRNA when edeine is added before the mRNA and <sup>35</sup>S methionine. When 2 $\mu$ M edeine is added 2 to 5 min before mRNA, all stimulation is abolished. The fact that all stimulation can be inhibited implies that all the stimulation requires peptide chain initiation.

Experiments were then designed to determine the length of time for which initiation is possible. Two alternative approaches were considered.

Firstly, the time at which edeine could be added and no longer cause inhibition would be the time when no new initiation was occurring. However, this would be affected by the stability of mRNA, because the absence of initiation could reflect the absence of intact initiation sites.

The alternative approach is to add mRNA and label at different times after the incubation is started, and to look for stimulation over the endogenous rate. Because all stimulation requires initiation (Fig. 35b), the time when added mRNA does not stimulate is the time when initiation is no longer occurring. The results shown before (controls in Fig. 35b) suggested that this approach would be successful, and that about 50% of

FIG.35. Protein synthesis initiation in detergent extracts

(a,b) Effect of edeine on protein synthesis. Different extracts were used.

(a) 50  $\mu$ l incubations containing 99  $\mu$ g extract protein 10.3  $\mu$ Ci  $^{35}$ S methionine, 0 or 2  $\mu$ g SFV mRNA, and other components for protein synthesis, were incubated at 30°C with 0, 1 or 2  $\mu$ M edeine. 5  $\mu$ l aliquots were taken as required for acid precipitation.

0 $\mu$ M edeine	○ endogenous and	● SFV mRNA stimulated
1 $\mu$ M	□	■
2 $\mu$ M	△	▲

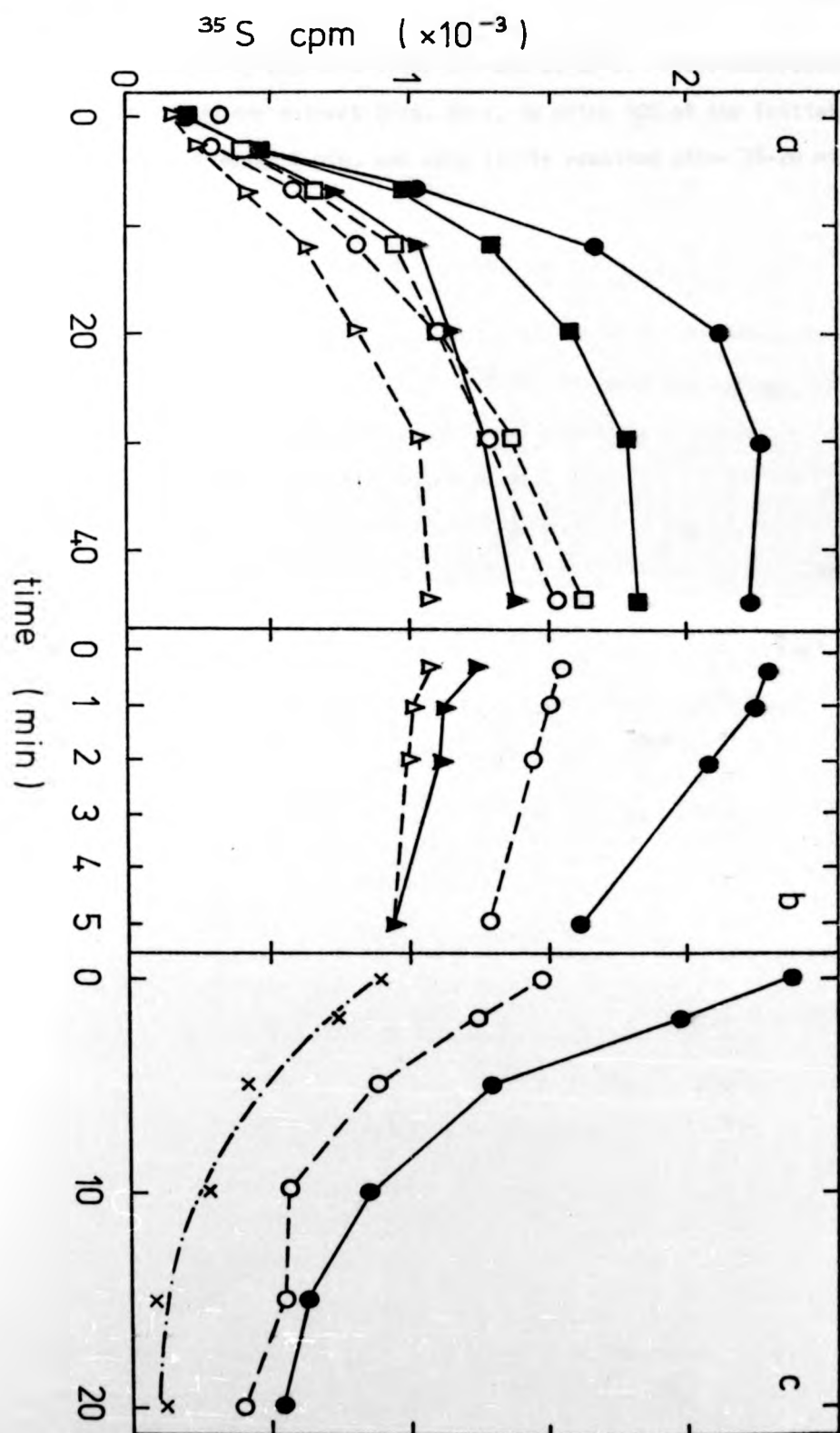
(b) 100  $\mu$ l incubations containing 202  $\mu$ g extract protein and other components for protein synthesis, but lacking  $^{35}$ S methionine and mRNA, were incubated at 30°C. At 2 min, edeine was added to 0, 1 or 2  $\mu$ M and incubation continued. After a further 15, 60, 120 or 300 s, 10  $\mu$ l aliquots were removed to vials containing 1.8  $\mu$ l of  $^{35}$ S methionine and water or SFV mRNA. These incubations were continued for a further 30 min, when two 3  $\mu$ l aliquots were taken for acid precipitation. These final incubations contained 440  $\mu$ Ci/ml  $^{35}$ S methionine and 0 or 40  $\mu$ g/ml SFV mRNA. Incorporation is plotted against the time between addition of edeine and addition of isotope and mRNA, for 0 and 2  $\mu$ M edeine incubations, the 1  $\mu$ M edeine results were intermediate between these.

0 $\mu$ M edeine;	○ endogenous and	● SFV mRNA stimulated
2 $\mu$ M	△	▲

(c) Effect of time of preincubation before adding isotope and mRNA on stimulation of protein synthesis.

A 400  $\mu$ l incubation containing 582  $\mu$ g extract protein and other components for protein synthesis, but lacking  $^{35}$ S methionine and mRNA, was incubated at 30°C. At various times, 21.5  $\mu$ l aliquots were removed to vials containing 4.5  $\mu$ l of  $^{35}$ S methionine and water or SFV mRNA. These incubations were continued for a further 30 min, when two 3  $\mu$ l aliquots were taken for acid precipitation. These final incubations contained 440  $\mu$ Ci/ml  $^{35}$ S methionine and 0 or 40  $\mu$ g/ml SFV mRNA. Incorporation is plotted against the time of preincubation before the addition of isotope and mRNA.

○ endogenous
● SFV mRNA stimulated
× stimulation over endogenous





the initiation capacity was lost after 6-7 min at 30°C. These observations were repeated in another extract (Fig. 35c), in which 50% of the initiation capacity decayed in about 5 min, and very little remained after 15-20 min.

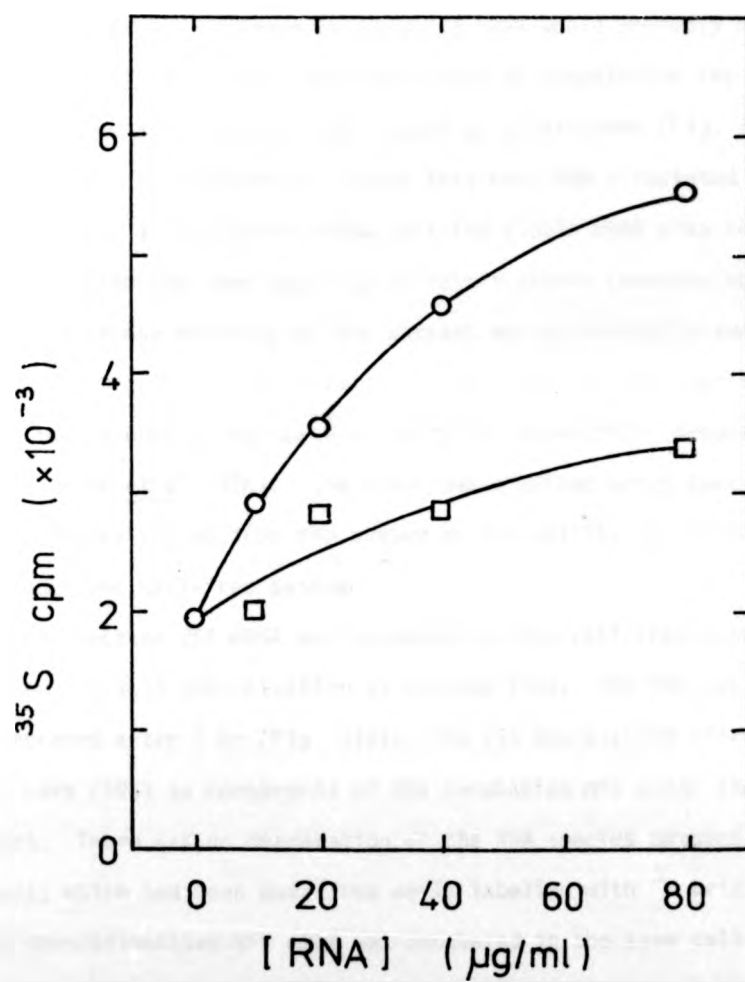


FIG.36. Effect of E.coli tRNA on protein synthesis

50  $\mu$ l incubations containing 91  $\mu$ g extract protein, 39  $\mu$ Ci  $^{35}$ S methionine, various concentrations of globin mRNA or E. coli tRNA, and other components for protein synthesis, were incubated at 30°C for 30 min before taking two 5  $\mu$ l aliquots for acid precipitation. The remainder of each incubation was electrophoresed on a gradient gel (results not shown).

○ + globin mRNA

□ + E.coli tRNA



#### 6.6. RIBONUCLEASE ACTIVITY

Although the stimulation by added mRNA required new peptide chain initiation (Fig. 35b), it was partly due to increased translation of endogenous mRNA (Fig. 34). To check for effects which were not specific for mRNA, the stimulation due to E.coli tRNA was compared to that due to globin mRNA. Any effect of added prokaryotic tRNA would probably be non-specific (Aviv et al 1971), and could be caused by competition for nucleases. The stimulation was about 35% of that caused by globin mRNA (Fig. 36). Polyacrylamide gel electrophoresis showed that the tRNA stimulated the same endogenous products as did globin mRNA, but the globin mRNA also coded for a product running with the same mobility as rabbit globin (results not shown).

The ribonuclease activity of the extract was estimated by two different methods. One was the acid precipitation method used by other workers to demonstrate the absence of nuclease activity in conventionally prepared cell-free systems (Kerr et al 1976). The other was a method which measured the integrity of RNA extracted from the system by its ability to be translated in a mRNA-dependant cell-free system.

When radioactive 26S mRNA was incubated in the cell-free extract, and samples taken for acid precipitation at various times, the RNA was less than 35% degraded after 1 hr (Fig. 37a). The 25% degradation after 30 min was due in part (10%) to components of the incubation mix other than the cell extract. There was no degradation of the RNA species present in the cell extract, which had been made from cells labelled with  $^3\text{H}$  uridine.

When non-radioactive SFV mRNA was incubated in the same cell-free extract, and samples taken for RNA isolation and translation in the mRNA-dependant reticulocyte lysate (Pelham and Jackson 1976), a different picture emerged (Fig. 37b). As the incubation proceeded, the extracted RNA was less efficient at stimulating the lysate. The activity dropped to the level produced by RNA extracted from an incubation containing only endogenous RNA.

FIG.37. Ribonuclease activity in detergent extracts

The same extract, from cells which had been labelled with 5  $\mu\text{Ci/ml}$   $^3\text{H}$  uridine between 50 hr and 20 hr before extraction, was used for both experiments. It contained  $6 \times 10^5$   $^3\text{H}$  cpm/ml.

(a) Degradation of labelled mRNA in the extract.

$^{32}\text{P}$  labelled 26 S mRNA (kind gift of S.I.T. Kennedy) was added to 25  $\mu\text{l}$  incubations at about  $10^4$  cpm per assay. The incubations also contained 45  $\mu\text{g}$  of extract protein ( $10^4$   $^3\text{H}$  cpm), 1  $\mu\text{g}$  unlabelled SFV mRNA, and other components for protein synthesis, including 50  $\mu\text{M}$   $^{32}\text{S}$  methionine. After various times at  $30^\circ\text{C}$ , duplicate 7.5  $\mu\text{l}$  aliquots were taken for acid precipitation. The results are plotted as a percentage of the zero time control (3050 cpm) against time of digestion. A background of 930 cpm, for a sample incubated with 0.4 mg/ml RNase A and  $3 \times 10^4$  u/ml RNase T1 for 30 min, has been subtracted from all samples. There was no reduction in the  $^3\text{H}$  cpm.  $\circ$  complete system;  $\Delta$  no cell extract;  $\square$   $^{32}\text{P}$  26S mRNA incubated alone.

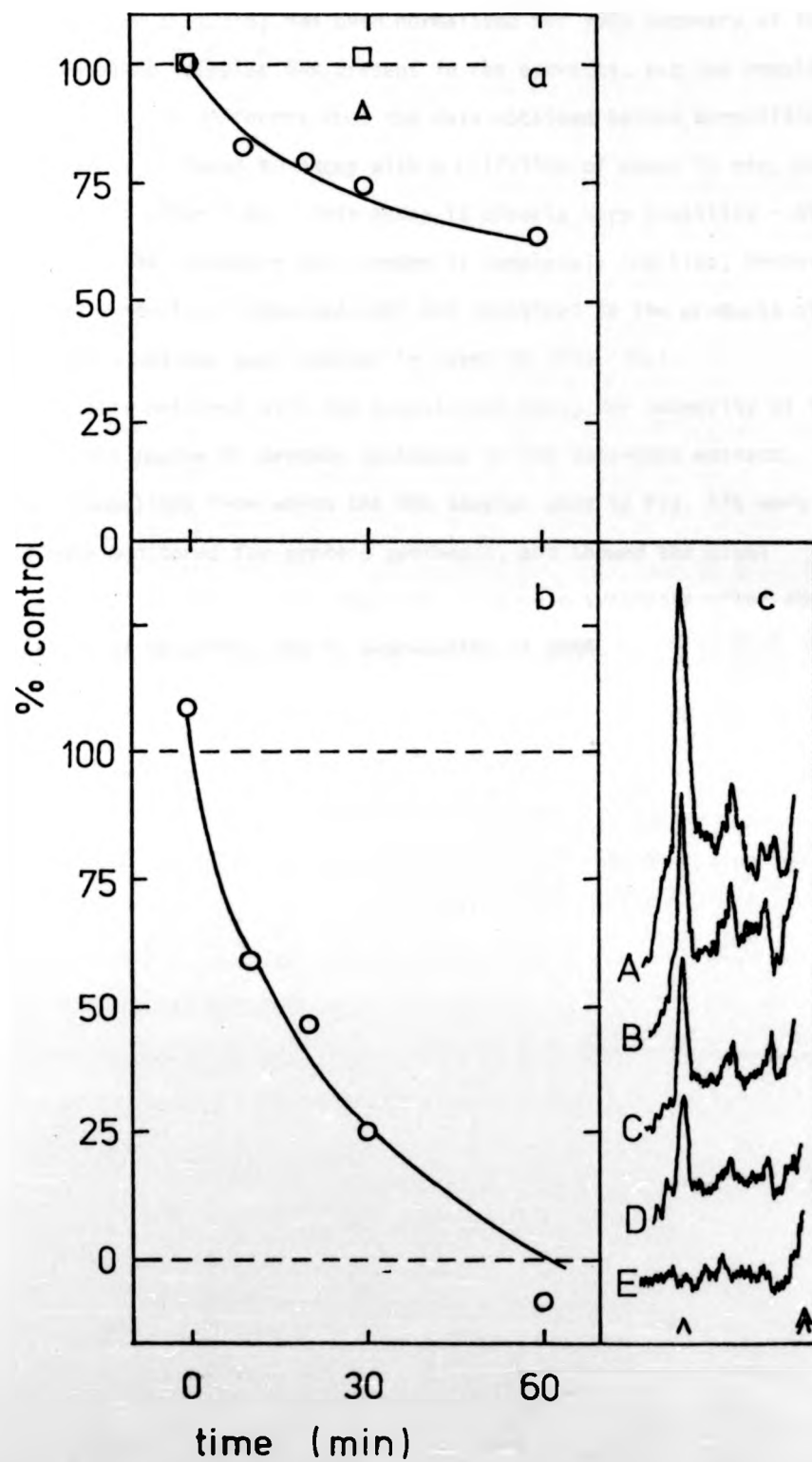
(b,c) Degradation of unlabelled mRNA in the extract.

Two incubations, one of 375  $\mu\text{l}$  and one of 150  $\mu\text{l}$ , were prepared, containing SFV mRNA at 40 or 0  $\mu\text{g/ml}$  respectively, and other components for protein synthesis. The kinetics of protein synthesis were normal (data not shown). 50  $\mu\text{l}$  aliquots were extracted with phenol and chloroform as described (section 2.4.5). The RNA was precipitated in the presence of carrier *E. coli* tRNA and assayed for stimulation of the micrococcal nuclease treated reticulocyte lysate (section 2.3.3). 50  $\mu\text{l}$  incubations contained 10  $\mu\text{l}$  of extracted RNA or control RNA, and 0.9  $\mu\text{Ci}$  of  $^{35}\text{S}$  methionine. After 30 min at  $30^\circ\text{C}$ , two 5  $\mu\text{l}$  aliquots were taken for acid precipitation and the remainder prepared for gel electrophoresis (section 2.6.2).

(b) Stimulation of the micrococcal nuclease treated reticulocyte lysate corrected for efficiency of RNA extraction (60-80%), related to stimulation by 400 ng SFV mRNA (100% = 33000 cpm/5  $\mu\text{l}$ ) and stimulation by RNA extracted from endogenous incubation (0% = 6000 cpm/5  $\mu\text{l}$ ).

Background (i.e. no added RNA) for this lysate was 4000 cpm/5  $\mu\text{l}$ , and it was linearly dependant on added SFV mRNA (data not shown).

(c) Products of micrococcal nuclease treated lysate analysed on a 12.5% polyacrylamide gel (section 2.6.2). Densitometer scans are shown for A, RNA extracted from the SFV mRNA incubation after 0 min; B, after 10 min; C, after 20 min; D, after 30 min; and E after 60 min. The markers are SFV capsid protein (single arrow) and the top of the resolving gel (double arrow).



The data presented (Fig. 37b) has been normalised for 100% recovery of the  $^3\text{H}$  uridine-labelled cellular RNA present in the extracts, but the results are not substantially different from the data obtained before normalising. The mRNA activity is found to decay with a half-life of about 15 min, and is fully degraded after 1 hr. This assay is clearly very sensitive - minor changes to the mRNA structure must render it completely inactive, because the cell-free products of degraded mRNA are identical to the products of undegraded mRNA, and are just reduced in quantity (Fig. 37c).

The results obtained with the translation assay for integrity of the mRNA reflect the course of protein synthesis in the detergent extract. The particular incubations from which the RNA samples used in Fig. 37b were extracted were monitored for protein synthesis, and showed the usual kinetics (as in Fig. 30a). The reduction in protein synthesis after about 20 min could thus be partly due to degradation of mRNA.

#### 6.7. PROTEIN KINASE ACTIVITY

In view of the possible involvement of protein phosphorylation in the control of translation (Ernst *et al* 1976), transfer of the  $\gamma$  phosphate of ATP into proteins was analysed. Some of these experiments were performed under conditions where protein synthesis was not characterised, for example, at 37°C and in the absence of the usual components for protein synthesis.

Fig. 38a shows the kinetics of  $^{32}\text{P}$  incorporation from ATP  $\gamma$   $^{32}\text{P}$  into acid insoluble material. The incorporation is maximal at about 5 min, the subsequent decrease implying phosphatase activity. Fig. 38b,c shows that even though cold acid precipitates more radioactivity than hot acid, all of the cold (and hot) acid insoluble material is pronase sensitive yet DNase and RNase insensitive. This implies that all the incorporated label is bound to protein, some being released by hot acid. This was corroborated by phenol extraction of the incubation mixture - the radioactivity recovered from the phenol layer by acetone precipitation equalled the hot acid insoluble radioactivity present (data not shown).

When the incubation mixture was treated with SDS, reduced with  $\beta$  mercaptoethanol, and fractionated on SDS polyacrylamide gels, many discrete bands were seen (for example, Fig. 47). These bands remained even when the gel was boiled in TCA (Auerbach and Pederson 1975).

The implication that this material represents phosphate covalently bound to proteins could be confirmed by showing that all the acid insoluble radioactivity is contained in phosphorylated aminoacid residues.



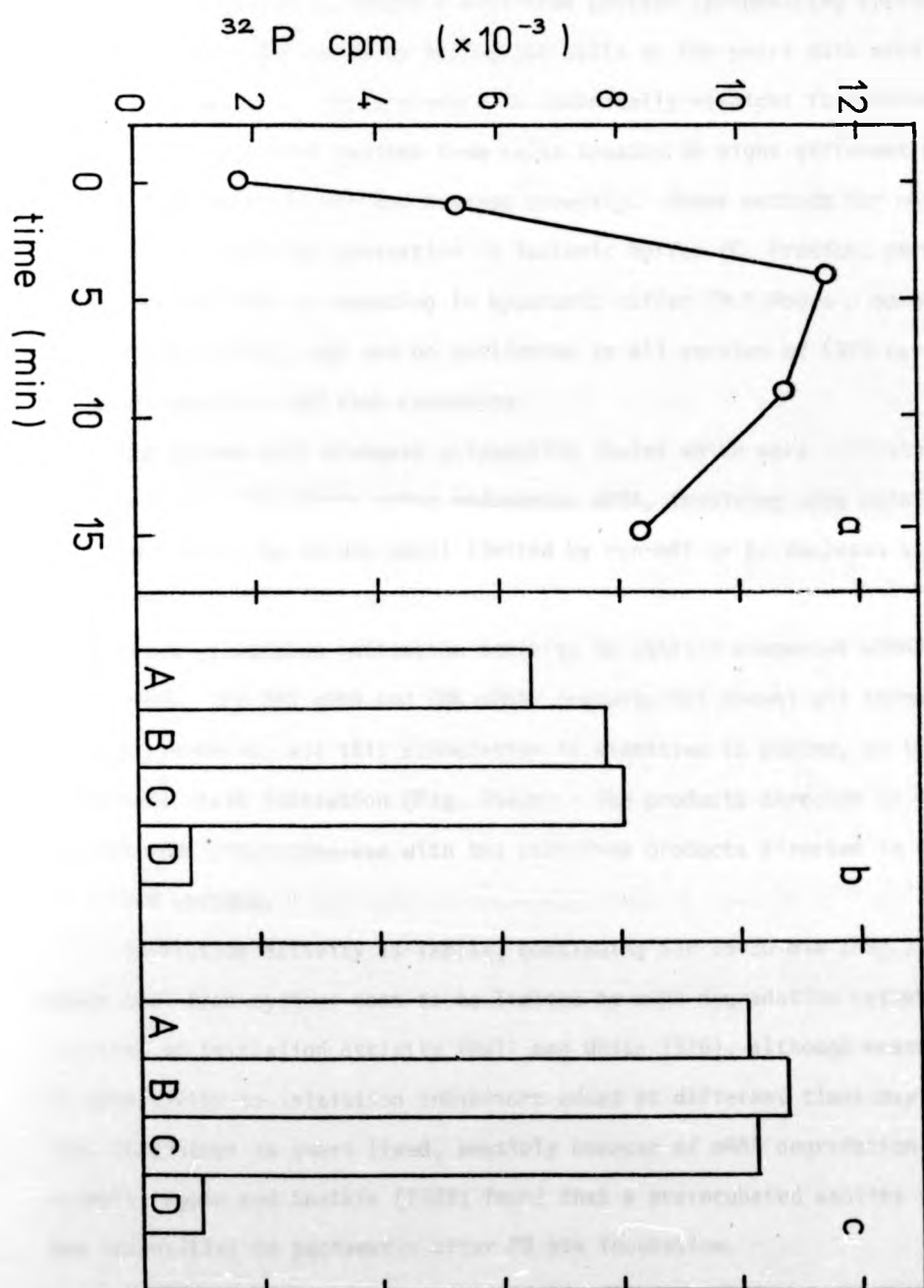


FIG. 38. Kinetics of incorporation of  $^{32}\text{P}$  and the nature of the acid insoluble product.

A detergent extract was made, and assayed without storage, according to the procedure described (section 2.3.7). 100  $\mu\text{l}$  incubations contained 90  $\mu\text{l}$  of extract and 10  $\mu\text{l}$  of about 20 mCi/ml ATPY  $^{32}\text{P}$ . 5  $\mu\text{l}$  aliquots were taken for acid precipitation as required, and at 15 min 5  $\mu\text{l}$  aliquots were taken to 1 ml DNase buffer (2 mM Mg (OOCCH<sub>3</sub>)<sub>2</sub>, 50 mM NaCl, 10 mM tris HCl pH 7.5) or 1 ml tris buffer (50 mM tris HCl pH 7.5). These were treated with DNase at 0 or 50  $\mu\text{g}/\text{ml}$  (in DNase buffer), or with 100  $\mu\text{g}/\text{ml}$  RNase A + 1000 u/ml RNase T1 (in tris buffer), or with 100  $\mu\text{g}/\text{ml}$  pronase (in tris buffer), at 37°C for 30 min before making 10% with TCA and filtering.

(a) Time course of  $^{32}\text{P}$  incorporation into hot acid insoluble cpm at 37°C.

(b and c) Results of enzyme treatments on acid insoluble  $^{32}\text{P}$

(b) hot acid insoluble cpm.

(c) cold acid insoluble cpm.

In each case the treatments were: A; controls (DNase buffer or tris buffer); B, DNase treated; C, RNase treated; D, pronase treated.

time (min)

#### 6.8. DISCUSSION

It is possible to obtain a cell-free protein synthesising system from monolayer grown L929 cells by lysing the cells on the petri dish with a non-ionic detergent. The procedure is technically straight forward and very rapid - cell-free systems from cells treated in eight different ways can be prepared in 30 min and assayed directly. Other methods for monolayer grown cells, involving sonication in isotonic buffer (C. Preston, personal communication 1976) or douncing in hypotonic buffer (N.F. Moore, personal communication 1976), may not be applicable to all strains of L929 cells and are more laborious and time consuming.

The system will elongate polypeptide chains which were initiated before cell breakage. Synthesis using endogenous mRNA, involving some reinitiation, continues for up to 30 min until limited by run-off or by nuclease activity (Fig. 30a).

There is surplus initiation activity to utilise exogenous mRNAs. Globin mRNA, SFV 26S mRNA and EMC mRNA (results not shown) all stimulate protein synthesis, and this stimulation is sensitive to edeine, an inhibitor of peptide chain initiation (Fig. 35a,b). The products directed by globin and 26S mRNA electrophorese with the cell-free products directed in other cell-free systems.

Initiation activity is labile, continuing for 15-20 min (Fig. 35c). Other cell-free systems seem to be limited by mRNA degradation rather than survival of initiation activity (Ball and White 1976), although measurement of sensitivity to initiation inhibitors added at different times may imply that initiation is short lived, possibly because of mRNA degradation. For example, Eggen and Shatkin (1972) found that a preincubated ascites system was insensitive to pactamycin after 20 min incubation.

Various modifications were tried to extend the life of the system (data not shown). Addition of reticulocyte ribosomal salt wash factors, prepared

according to Schreier and Staehelin (1973b; see section 2.3.10), and either added crude or as separate fractions A and B1, had no beneficial effects on SFV mRNA-stimulated incorporation. Addition of hemin to 50 $\mu$ M (Weber *et al* 1975) or glucose to 2mM (R.J. Jackson, personal communication 1975; Giloh and Mager 1975), was not stimulatory. Lowering the magnesium concentration by 1.4mM and replacing it with 80 $\mu$ M spermine (Atkins *et al* 1975) did not promote SFV mRNA translation. It is possible, however, that the effects of some of these treatments were masked by other changes in the system, and probably a better test for prolonged initiation would be to assay for ability to utilise mRNA added after, say, 30 min of incubation.

The presence of non-ionic detergent in the system does not seem to be detrimental. Addition of extra detergent does not inhibit protein synthesis, and other cell-free systems are active under these conditions. The reticulocyte lysate is inhibited only 25% by 0.08% v/v Triton N101 (the maximum concentration present in assays using the detergent extract) and the kinetics suggest progressive deterioration of the system rather than specific inhibition of initiation (data not shown). Recently, Briendl and Holland (1976) have published evidence that the ascites and wheat germ systems are inhibited 25% by 0.1% v/v Nonidet P40.

The detergent may have an indirect effect on protein synthesis by releasing lysosomal ribonucleases and proteases. Protease activity was probably only slight. More important was the ribonuclease activity, which destroyed all active mRNA in 60 min (Fig. 37b). However, the nuclease activity measured by acid precipitation of radioactive mRNA (Fig. 37a), compares favourably with published data (Kerr *et al* 1976). The sedimentation profiles of reovirus mRNA presented by Graziadei *et al.* (1973a) showed 50% degradation after about 15 min in their L929 cell system. It is possible that the effects of nuclease activity could be reduced by assaying protein synthesis in the presence of *E.coli* tRNA (Fig. 37), 5'CMP (Cuatrecasas *et al* 1967) or EGTA (Pelham and Jackson 1976).

The relationship between protein concentration and protein synthesis (Fig. 30b) implies that more concentrated extracts are desirable. G. Thomas (personal communication 1976) has shown that the supernatant from WI38 fibroblasts, lysed on the petri dish with 1% Triton X-100, contains only a small proportion of the total polysomes, the remainder being strongly attached to the nuclear membrane and cytoskeleton. D. Levin has suggested a modification to the basic procedure, in which the cells are scraped off the dish, pelleted at low speed, resuspended in one packed-cell volume of isotonic buffer, and lysed with the addition of detergent to 0.1%. The nuclei are spun out at low speed and the supernatant assayed. In my hands this protocol produced extracts with 5-6 times the protein concentration of the standard extracts. Protein synthesis was about 3 times that of the standard extracts, which, considering the pool concentration of methionine is probably 5-6 times higher, represents a considerable improvement. However, the kinetics of synthesis were similar, implying that short-lived initiation and high nuclease activity were still a problem.

The benefits of the detergent procedure arise from its speed and ease of handling, enabling comparison of in vivo and in vitro changes under a range of conditions. The procedure may find application in the study of control of translation under conditions of growth control, virus-induced shut off, and interferon action (section 7). The method has been used, without modification, to obtain an active cell free system from monolayer grown Hela cells (results not shown).

It is interesting that, when the endogenous synthesis by 25 extracts was surveyed, the synthesis rate was found to depend on the history of the cells. Extracts of cells grown overnight in 2% serum were only one third as active as cells grown in 10% serum (results corrected for protein concentration; results significant at 99% level of confidence). Whether this arises from differences in pool sizes, availability of mRNA, or activity of the ribosomes, is not known. Hassell and Engelhardt (1973) reported a similar difference in cell-free elongation systems from Vero cells.

SECTION 7  
CORRELATION OF IN VIVO AND IN VITRO  
EFFECTS OF INTERFERON AND dsRNA

## 7. CORRELATION OF IN VIVO AND IN VITRO EFFECTS OF INTERFERON AND dsRNA

### 7.1. INTRODUCTION

Cell-free systems from interferon-treated cells are highly sensitive to dsRNA (Kerr et al 1974b). Unlike the other effects observed in vitro (Falcoff et al 1972, Content et al 1974, Gupta et al 1974, Samuel and Joklik 1974), the dsRNA effect is found in non-preincubated cell-free systems, and is thus unlikely to be an artefact of preincubation or gel filtration (Sen et al 1976). Inhibition by dsRNA could be the basis for the antiviral effect of interferon, because some cytoplasmic dsRNA is produced during replication of most viruses, and this could trigger a translation block and prevent viral multiplication (Kerr et al 1973, Kerr et al 1974b). Indeed, cell free systems from interferon-treated, infected cells, show the same characteristics as interferon-treated cell extracts which have been incubated with dsRNA (Friedman et al 1972a,b).

The in vitro effect of dsRNA might also be involved in the increased toxicity of dsRNA towards interferon-treated cells (Stewart et al 1972a). If dsRNA entered the cells and inhibited protein synthesis, toxicity could result either directly, or from the accumulation of some regulatory component. Alternatively, the translational inhibitor might be capable of other effects in vivo, for example, by causing membrane disintegration. Thus, even though no evidence for gross inhibition of protein synthesis early in the cytotoxic effect has been found (section 5.6), it was still possible that the translational inhibitor might be present, and might correlate with the development of toxicity.

Another system which is highly sensitive to dsRNA is the reticulocyte lysate, in which the inhibitor acts at initiation (Darnbrough et al 1972). In this system, the inhibition is probably due to a protein kinase, which phosphorylates an initiation factor (Ernst et al 1976, P. Farrell, K. Balkow, T. Hunt, R. Jackson, personal communication 1975). For this reason, the experiments described here were designed with two aims in view.

Firstly, to establish an assay for the interferon and dsRNA dependant inhibitor, to characterise it and compare it with the reticulocyte inhibitor, and to compare its formation in vivo and in vitro. Secondly, to search for changes in patterns of protein phosphorylation which might correlate with the cytotoxic effect.

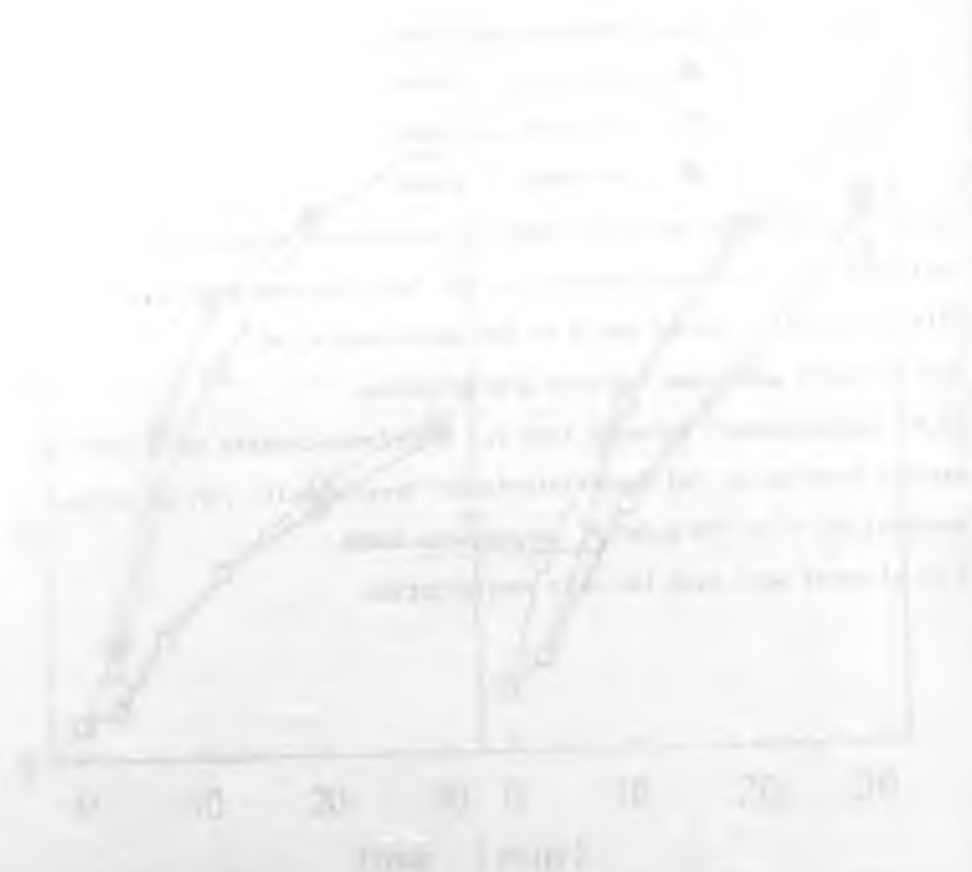




FIG.39. Effect of interferon and dsRNA on in vitro protein synthesis

140 mm petri dish cultures of cells were treated for 17 hr with 200 u/ml interferon, or an equivalent concentration of "mock-interferon", in GMEM/2% CS. The cells were then washed and detergent extracts made as before (section 2.3.5) or by scraping the cells into conical centrifuge tube, pelleting at 3750g, adding an equal volume of 90 mM KCl, 30 mM Hepes KOH, 1mM DTT, 4mM Mg(OOCCH<sub>3</sub>)<sub>2</sub>, pH 7.4, making 0.1% v/v in Triton N101, douncing gently, and taking a 4200g supernatant (D. Levin, personal communication 1976). Two different batches of cells were used, one for the "conventional" extracts, and one for the "concentrated" extracts, and they were assayed on separate occasions. 50 µl incubation mixtures contained 35 µl of extract, 17.8 µCi <sup>35</sup>S methionine, 0 or 40 µg/ml SFV mRNA, and dsRNA as required. Aliquots were withdrawn as required for acid precipitation.

○ endogenous, no dsRNA

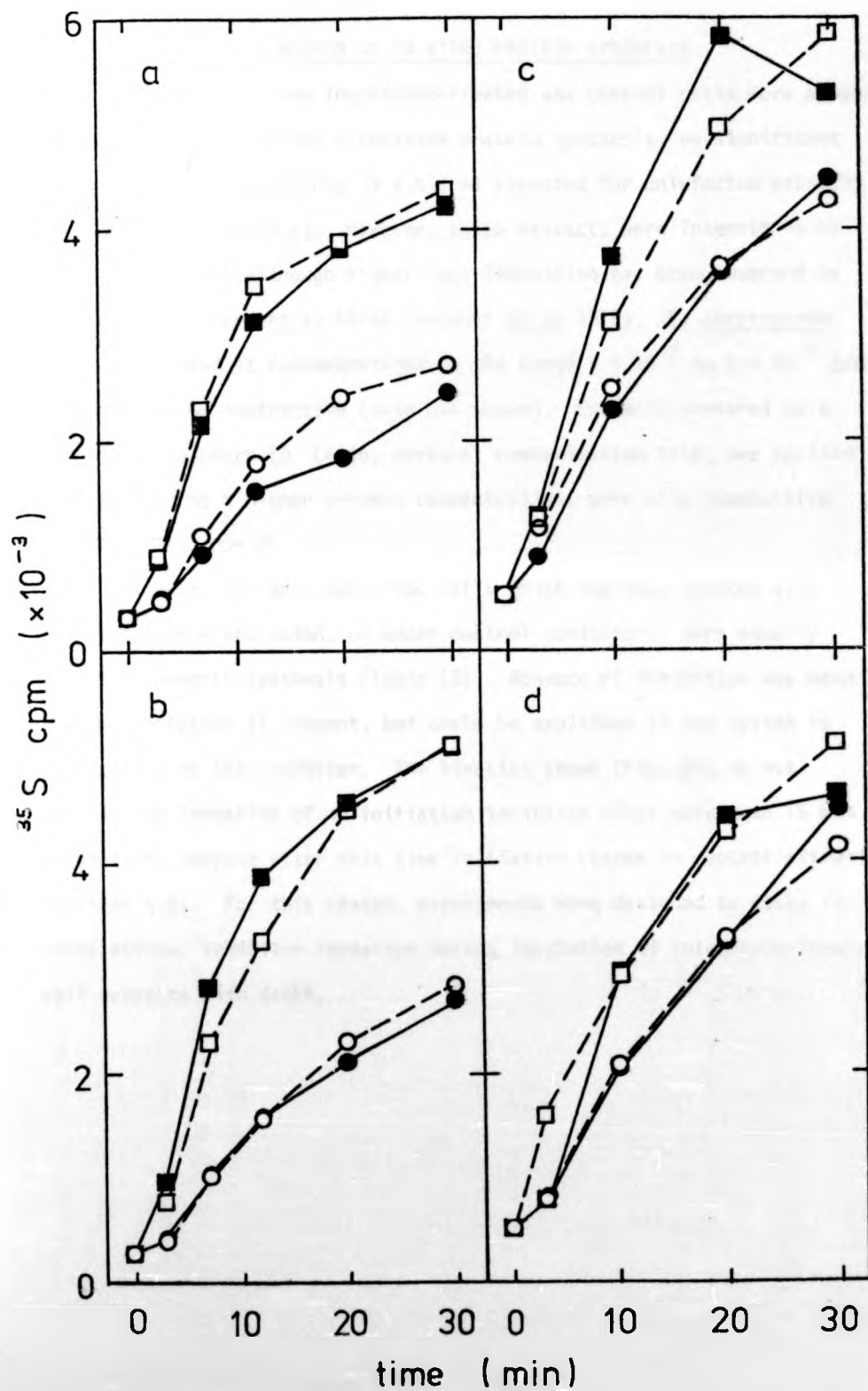
● endogenous, + dsRNA

□ + SFV mRNA, no dsRNA

■ + SFV mRNA, + dsRNA

(a,b) "conventional" extracts from (a) interferon-treated cells (assays contained 133 µg extract protein) or (b) "mock-interferon"-treated cells (114 µg extract protein) and 0 or 250 ng/ml poly I.rC. 5 µl aliquots were used for acid precipitation.

(c,d) "concentrated" extracts from (c) interferon-treated cells (231 µg extract protein) or (d) "mock-interferon" treated cells (212 µg extract protein) and 0 or 100 ng/ml P. chrysogenum dsRNA. 3 µl aliquots were used for acid precipitation.



## 7.2. EFFECTS OF INTERFERON ON IN VITRO PROTEIN SYNTHESIS

When extracts from interferon-treated and control cells were assayed for endogenous or SFV RNA stimulated protein synthesis, no significant differences were found (Fig 39 a,b), as expected for uninfected extracts (Friedman et al 1972a,b). However, these extracts were insensitive to dsRNA (Fig 39a,b) although significant inhibition has been observed by other workers (Kerr et al 1974b, Content et al 1975). P. chrysogenum dsRNA was tested at concentrations in the range  $5 \times 10^{-8}$  to  $5 \times 10^{-5}$  g/ml, and found to be ineffective (data not shown). Extracts prepared by a different procedure (D. Levin, personal communication 1976, see section 6.8) and having a higher protein concentration, were also insensitive to dsRNA (Fig. 39c,d).

Moreover, extracts made from cells which had been treated with both interferon and dsRNA, or under control conditions, were equally active in protein synthesis (Table 10). Absence of inhibition may mean that no inhibitor is present, but could be explained if the system is insensitive to the inhibitor. The kinetics shown (Fig. 39) do not exclude the formation of an initiation inhibitor after more than 15 min incubation, because after this time initiation ceases in control extracts (section 6.5). For this reason, experiments were designed to assay for translational inhibitor formation during incubation of interferon-treated cell extracts with dsRNA.

TABLE 10: Effect of treatment of cells on in vitro protein synthesis

Expt. No.	Treatment of cells			Cell-free system		
	Interferon (u/ml)	poly rI.rC ( $\mu$ g/ml)	% loss of protein at 3.5 hr <sup>b</sup>	concentration ( $\mu$ g/ml)	activity (cpm/ $\mu$ g protein)	
					endogenous	+ SFV mRNA (40 $\mu$ g/ml)
1.	100	50	23	2.78	247	460
	100	0	-	3.76	254	451
	0 <sup>a</sup>	50	-	3.14	215	491
	0 <sup>a</sup>	0	-	4.20	198	399
2.	1000	50	41	2.06	198	279
	300	50	49	1.79	137	224
	100	50	47	1.89	147	236
	30	50	39	1.53	172	247
	100	0	-	1.59	204	310
	0 <sup>a</sup>	50	-	2.09	262	331

Duplicate 140 mm petri dish cultures were treated with interferon for 18 hr and poly rI.rC as shown, and detergent extracts prepared. Extracts from duplicate plates were pooled and assayed for protein concentration and <sup>35</sup>S methionine incorporation (30 min at 30°C).

Expt. 1. Extracts made 30-35 min after poly rI.rC and assayed with 36  $\mu$ Ci <sup>35</sup>S methionine/50  $\mu$ l incubation Expt. 2. Extracts made 60-70 min after poly rI.rC and assayed with 18  $\mu$ Ci <sup>35</sup>S methionine/50  $\mu$ l incubation a) Treated with "mock-interferon". b) Parallel cultures in 25 mm vials were assayed in the usual way for

cytotoxic effects.

### 7.3. ASSAYS FOR A TRANSLATIONAL INHIBITOR

Interferon or mock-treated cell extracts, an energy supply, and poly rI.rC at 250 ng/ml, were incubated under conditions suitable for protein synthesis for 15 min, and then diluted ten-fold into fresh incubations containing control cell extract, energy supply and aminoacids. After 5 min incubation, radioactive methionine and SFV RNA were added, and acid insoluble radioactivity measured after a further 30 min. This protocol allowed 15 min for the formation of an inhibitor, and at least 5 min for its action, but even so, no inhibition of the stimulation by SFV RNA could be detected (data not shown).

It was decided to use a system with linear kinetics to assay for the production of inhibitor.

The reticulocyte lysate is linear for at least 30 min and initiation of new polypeptide chains continues through this period. However, the lysate is sensitive to small amounts of dsRNA, so any assay for a dsRNA-dependant inhibitor must use conditions where preformed inhibitor is active but the lysate inhibitor is not formed. High concentrations of dsRNA are not inhibitory (Legon *et al* 1974) but can only partly reverse the inhibition due to low amounts of dsRNA (Hunter *et al* 1975), and it is likely that high concentrations of dsRNA prevent the formation of the lysate inhibitor (Hunter *et al* 1975; P. Farrell, personal communication 1976).

Fig. 40b shows that dsRNA at 10 ng/ml inhibits the lysate with biphasic kinetics. However, at 1  $\mu$ g/ml or higher concentrations, the inhibition is much reduced (Fig. 40c). Thus an assay system containing 10  $\mu$ g/ml dsRNA is insensitive to the addition of low concentrations of dsRNA (Fig. 40a) and can be used for the assay of preformed inhibitor.

When cell extracts were incubated with 100 ng/ml dsRNA and an energy supply, and then assayed for their ability to inhibit the lysate in the presence of 10  $\mu$ g/ml dsRNA, the result depended on the source of

FIG.40. Reticulocyte lysate assay for protein synthesis inhibitor

(a) Lysate assay in the presence of 10  $\mu\text{g/ml}$  *P. chrysogenum* dsRNA and aliquots of various preincubations.

Preincubations contained cell-extract protein from interferon or "mock-interferon" treated cells (same preparations as in Fig. 39a,b), or extraction buffer alone, energy supply, and *P. chrysogenum* dsRNA at 0 or 100 ng/ml (see section 2.3.9).

After 30 min at 30°C, 5  $\mu\text{l}$  aliquots were taken to 50  $\mu\text{l}$  incubation mixtures containing reticulocyte lysate, 10  $\mu\text{g/ml}$  dsRNA, and components for protein synthesis, including  $^{35}\text{S}$  methionine at 36  $\mu\text{Ci/ml}$ . After various times at 30°C, 3  $\mu\text{l}$  aliquots were removed for acid precipitation.

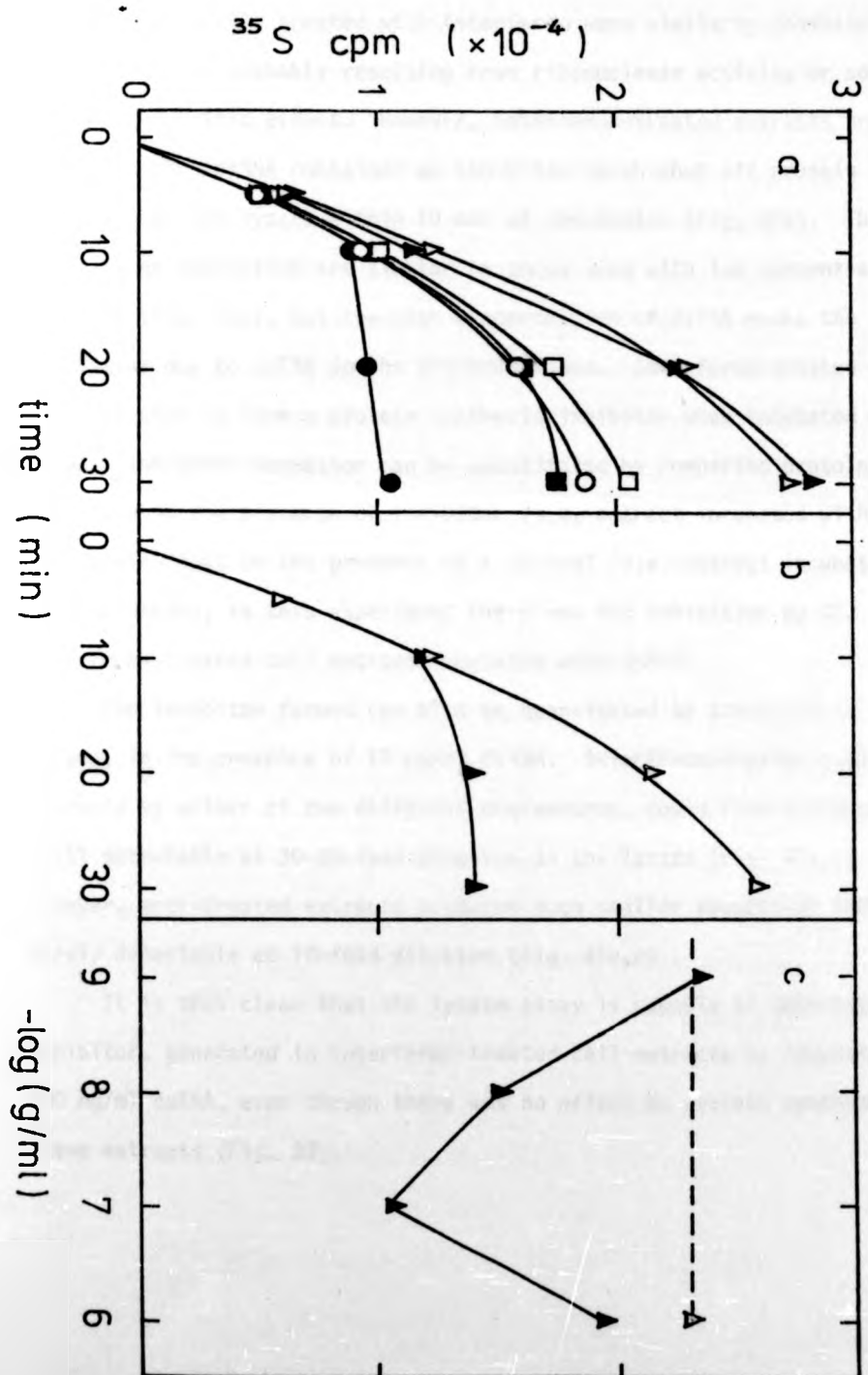
The preincubations contained

cell extract	dsRNA (ng/ml)	
buffer alone	0	▲
	100	▲
interferon	0	○
	100	●
"mock-interferon"	0	□
	100	■

(b) Lysate assay in the absence of high concentrations of dsRNA.

The "buffer alone" preincubations from part (a) were also assayed in an incubation containing no additional dsRNA. The final concentrations of dsRNA were thus: ▲ 0, and ▲ 10 ng/ml.

(c) Effect of various concentrations of *P. chrysogenum* dsRNA on protein synthesis. In a separate experiment, the lysate was incubated with various concentrations of dsRNA and 28  $\mu\text{Ci/ml}$   $^{35}\text{S}$  methionine. Duplicate 5  $\mu\text{l}$  aliquots were taken for acid precipitation after 30 min, and the incorporation is plotted against concentration of dsRNA. The broken line shows the incorporation in the absence of dsRNA.





extract (Fig. 40a). Extracts from cells treated with "mock-interferon" were somewhat inhibitory, whether incubated with or without dsRNA. Extracts from cells treated with interferon were similarly inhibitory, this inhibition probably resulting from ribonuclease activity or some other non-specific effect. However, interferon-treated extracts pre-incubated with dsRNA contained an inhibitor which shut off protein synthesis by the lysate within 10 min of incubation (Fig. 40a). The kinetics of inhibition are similar to those seen with low concentrations of dsRNA (Fig. 40b), but the high concentration of dsRNA masks the inhibition due to dsRNA in the preincubations. Interferon-treated extracts are thus able to form a protein synthesis inhibitor when incubated with dsRNA. Inhibitor formation can be quantitated by comparing protein synthesis in the presence of inhibitor (i.e. extract incubated with dsRNA) with that in the presence of a control (i.e. extract incubated without dsRNA); in this experiment there was 44% inhibition by the interferon-treated cell extract incubated with dsRNA.

The inhibitor formed can also be quantitated by titration in the lysate, in the presence of 10  $\mu\text{g/ml}$  dsRNA. Interferon-treated extracts, produced by either of two different procedures, could form inhibitor still detectable at 30-50 fold dilution in the lysate (Fig. 41a,c). However, mock-treated extracts produced much smaller amounts of inhibitor, barely detectable at 10-fold dilution (Fig. 41b,d).

It is thus clear that the lysate assay is capable of detecting an inhibitor, generated in interferon-treated cell extracts by incubation with 100 ng/ml dsRNA, even though there was no effect on protein synthesis in these extracts (Fig. 39).



FIG.41. Titration of dsRNA - dependant inhibitors

Inhibitors were formed in extracts of interferon and "mock-interferon" treated cells as described (section 2.3.9) and assayed, after dilution, in the presence of 10 µg/ml dsRNA (section 2.3.9). The assays contained 36 µCi/ml <sup>35</sup>S methionine, and 3 µl aliquots were removed after 30 min incubation for the determination of acid insoluble radioactivity. The incorporation in the presence of a given concentration of "inhibitor" (preincubation containing 100 ng/ml dsRNA) is expressed as a percentage inhibition, relative to the incorporation in the presence of a preincubation containing no dsRNA, and assayed at 100 µl/ml incubation (0% inhibition). The extracts used were the same as those described before (Fig.39).

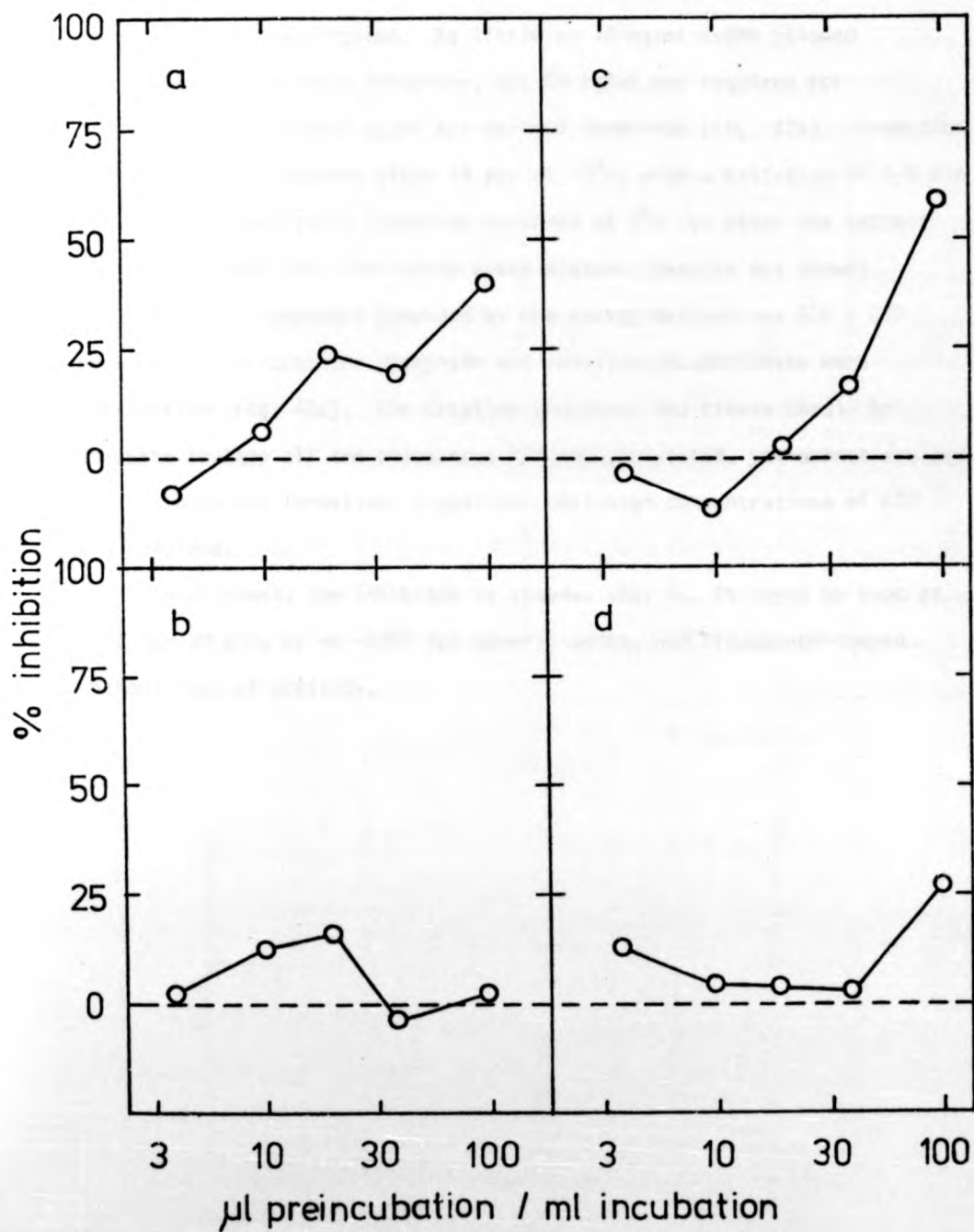
(a) "Conventional" extract from interferon-treated cells. The concentration of protein in the preincubations was 2.66 mg/ml and "0% inhibition" was 19900 cpm.

(b) "Conventional" extract from "mock-interferon" treated cells. The concentration of protein in the preincubations was 2.28 mg/ml and "0% inhibition" was 20000 cpm.

(c) "Concentrated" extract from interferon-treated cells. The concentration of protein in the preincubations was 4.62 mg/ml and "0% inhibition" was 19900 cpm.

(d) "Concentrated" extract from "mock-interferon" treated cells. The concentration of protein in the preincubations was 4.24 mg/ml and "0% inhibition" was 21400 cpm.

% inhibition



#### 7.4. REQUIREMENTS FOR INHIBITOR FORMATION

The requirements for forming the interferon and dsRNA dependant inhibitor were investigated. As little as 10 ng/ml dsRNA allowed formation of detectable inhibitor, but 20 ng/ml was required for half-maximal, and 40-80 ng/ml for maximal formation (Fig. 42a). Formation was essentially complete after 15 min at 30°C, with a half-time of 3-5 min (Fig. 42b). Negligible formation occurred at 0°C, or after the extract had been diluted into the lysate assay mixture (results not shown).

The only component provided by the energy mixture was ATP ; GTP or a mixture of creatine phosphate and creatine phosphokinase were ineffective (Fig. 42c). The creatine phosphate and kinase should be adequate to keep all the endogenous ATP phosphorylated, yet cannot replace ATP in inhibitor formation, suggesting that high concentrations of ATP are required.

Once formed, the inhibitor is stable; that is, it could be kept at 30°C for 30 min, or at -20°C for several weeks, and frozen-and-thawed without loss of activity.

FIG.42. Requirements for inhibitor formation.

Preincubations, containing "conventional" cell extract from interferon-treated cells, and *P. chrysogenum* dsRNA and energy supply as required (section 2.3.9), were performed for various times at 30°C before diluting 10-fold into reticulocyte lysate assays containing 10 µg/ml dsRNA (section 2.3.9) and <sup>35</sup>S methionine at 50.3 µCi/ml. After 30 min at 30°C, 3 µl aliquots were removed for acid precipitation. The incorporation in the presence of a given preincubation is expressed as a percentage inhibition, relative to the incorporation in the presence of a preincubation containing all the usual components but no dsRNA (0% inhibition = 28600 cpm).

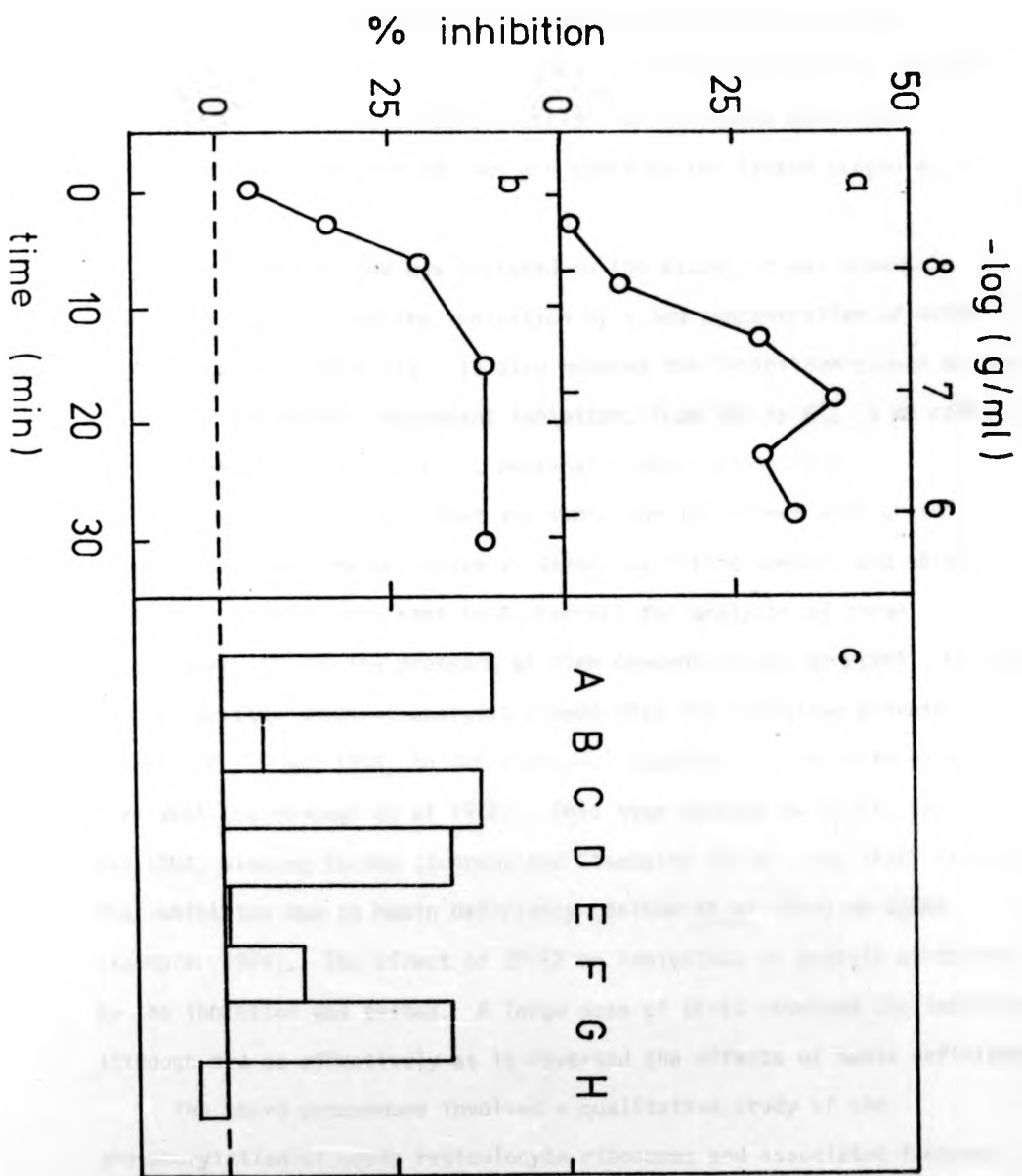
(a) Dependence on concentration of dsRNA. Percentage inhibition of the lysate is plotted against the log concentration of dsRNA present in the preincubation.

(b) Dependence on time of preincubation at 30°C. Aliquots of the preincubation were removed at various times and kept at 0°C until all the samples were ready for assay.

(c) Dependence on energy supply. The following components were present in the preincubation:

	creatine phosphate (mM)	creatine kinase (µg/ml)	ATP (mM)	GTP (mM)	CTP (mM)
A	10	25	1.0	0.1	0.6
B	10	25	0	0.1	0.6
C	10	25	1.0	0	0.6
D	0	0	1.0	0.1	0.6
E	0	0	0	0	0
F	10	25	0	0	0
G	0	0	2.0	0	0
H	0	0	0	2.0	0

time (min)



#### 7.5. ACTION OF THE INHIBITOR IN THE LYSATE ASSAY

The inhibitor was active in assays using three different reticulocyte lysates. The biphasic kinetics, with shut off as early as 5 min after the start of incubation, resembled the kinetics of inhibition by low concentrations of dsRNA, oxidising agents, or hemin deficiency (section 4.2). These three sets of conditions are all reversed when high concentrations of certain purines are added to the lysate (Legon *et al* 1974).

When 2-aminopurine was included in the assay, it was somewhat inhibitory, but reduced the inhibition by a low concentration of dsRNA from 66% to 20% (Table 11). It also reduced the inhibition caused by the interferon and dsRNA - dependant inhibitor, from 36% to 8%. 5 mM cAMP was also effective (P. Farrell, personal communication 1976).

It was thus possible that the inhibitor interfered with protein synthesis by the same mechanism as dsRNA, oxidising agents, and absence of hemin. Samples were sent to P. Farrell for analysis by three procedures, all in the presence of high concentrations of dsRNA. Firstly, sucrose density gradient analysis showed that the inhibitor prevents binding of  $^{35}\text{S}$  met tRNA<sub>F</sub> to 40S ribosomal subunits, as has been shown for dsRNA (Darnbrough *et al* 1972). This step depends on IF-E2, the met tRNA<sub>F</sub> binding factor (Schreier and Staehelin 1973a), and IF-E2 reverses the inhibition due to hemin deficiency (Balkow *et al* 1973) or dsRNA (Kaempfer 1974). The effect of IF-E2 on inhibition of protein synthesis by the inhibitor was tested. A large dose of IF-E2 reversed the inhibition, although not so effectively as it reversed the effects of hemin deficiency.

The third procedure involved a qualitative study of the phosphorylation of crude reticulocyte ribosomes and associated factors, under conditions where the factors are inactivated (Fig. 43). The  $\gamma$  phosphate of ATP is transferred to several proteins, but two proteins (molecular weights 35000 and 67000 daltons) are phosphorylated only in the presence of low concentrations of dsRNA or in the absence of hemin.

TABLE 11: Effect of 2-aminopurine on inhibition of the lysate

Expt. No.	Additions to the incubation				incorporation	
	dsRNA ( $\mu\text{g/ml}$ )	inhibitor	control	2-amino- purine (mM)	cpm	% inhibition
1.	10	-	+	0	5446	(0)
	10	+	-	0	4030	36
	10	-	+	7	4810	(0)
	10	+	-	7	4360	8
2.	0	-	-	0	49906	(0)
	0.01	-	-	0	16795	66
	0	-	-	7	43235	(0)
	0.01	-	-	7	34759	20

In Expt. 1. "inhibitor" (preincubation containing interferon-treated cell extract, energy supply, and 100 ng/ml dsRNA) or "control" (control cell extract, energy supply, and 100 ng/ml dsRNA) were assayed in the lysate, in the presence of 10  $\mu\text{g/ml}$  dsRNA and  $^{35}\text{S}$  methionine, at 30°C for 30 min.

In Expt. 2. inhibition by 10 ng/ml dsRNA was assayed in the same lysate, using  $^3\text{H}$  leucine at 30°C for 30 min.

The 35000 protein has been identified as the small subunit of IF-E2, and the 67000 protein is another ribosome-associated protein (P. Farrell, T. Hunt and R. Jackson, unpublished results). In the presence of the inhibitor, the 35000 protein was phosphorylated, and this was not prevented by high concentrations of dsRNA (Fig. 43). There was no phosphorylation when control incubations, containing no dsRNA, were added, and there was only partial phosphorylation when an incubation containing untreated cell extract and dsRNA was tested (Fig. 43). This is in agreement with the formation of small amounts of inhibitor by control extracts (Fig. 41b,d).

These results suggest that the inhibitor is a protein kinase, or a kinase activator, which can phosphorylate IF-E2 and thus create a lesion at polypeptide chain initiation.



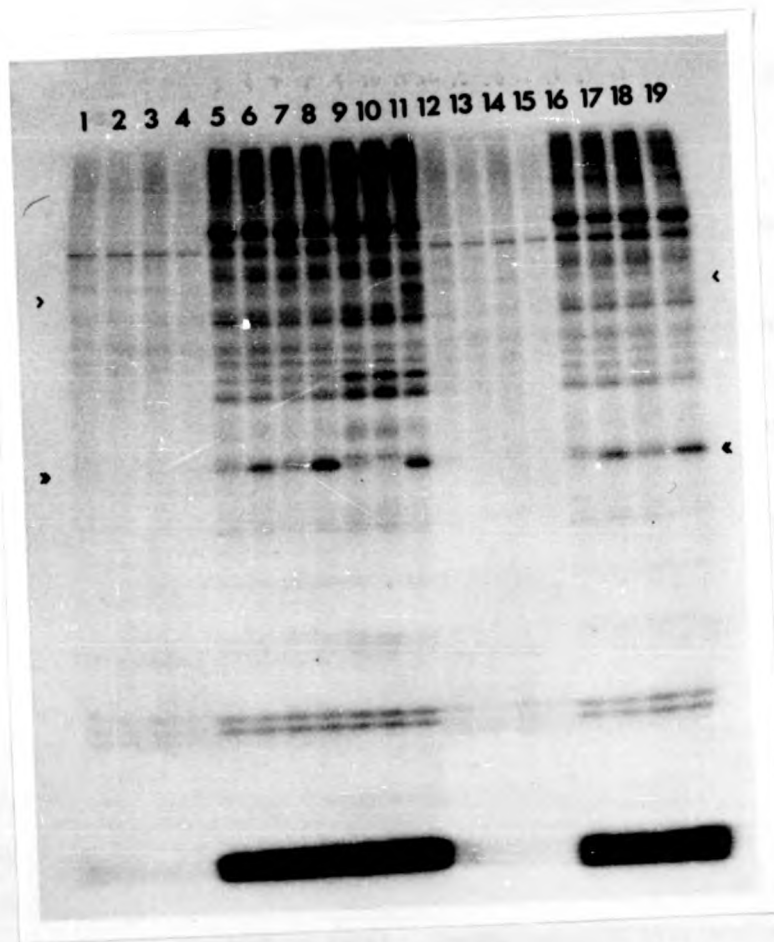
FIG.43. Phosphorylation of reticulocyte proteins by the inhibitor. \*

Preincubations containing interferon or control cell extracts, energy supply, and 0 or 100 ng/ml dsRNA were performed at 30°C for 30 min. Crude ribosomes were prepared from a hemin-treated lysate by gel-filtering on Sepharose 6B in a buffer containing 25 mM KCl, 10 mM NaCl, 1mM MgCl<sub>2</sub>, 0.25 mM DTT and 10 mM tris HCl pH 7.5. Phosphorylation assays contained, in 20 µl: 16 µl of ribosomes or buffer; 0, 0.05, or 20 µg/ml *P. chrysogenum* dsRNA; additional 100 mM KCl and 0.5 mM MgCl<sub>2</sub>; 50 µM ATP <sup>32</sup>P at 10 Ci/mmol; and 1 µl of each preincubation as required. Phosphorylation was allowed for 20 min at 30°C, before adding SDS and analysing the products on a 15% polyacrylamide gel (section 2.6.2). The gel was fixed, stained, and autoradiographed overnight. The tracks contained:

	preincubations				ribosomes	dsRNA
	control		interferon			(ug/ml)
	- dsRNA	+ dsRNA	- dsRNA	+ dsRNA		
1	+	-	-	-	-	20
2	-	+	-	-	-	20
3	-	-	+	-	-	20
4	-	-	-	+	-	20
5	+	-	-	-	+	20
6	-	+	-	-	+	20
7	-	-	+	-	+	20
8	-	-	-	+	+	20
9	-	-	-	-	+	0
10	-	-	-	-	+	20
11	-	-	-	-	+	0.05
12	+	-	-	-	-	0
13	-	+	-	-	-	0
14	-	-	+	-	-	0
15	-	-	-	+	-	0
16	+	-	-	-	+	0
17	-	+	-	-	+	0
18	-	-	+	-	+	0
19	-	-	-	+	+	0

The proteins which are phosphorylated in track 11 but not in tracks 9 and 10 are at 67000 daltons (single arrow) and 35000 daltons (double arrow), and the 35000 daltons protein is identical to the small subunit of IF-E2 (data not shown). Autoradiography of the gel for longer periods showed that there was no interferon and dsRNA specific phosphorylation in tracks 1-4 or 12-15.

\* Experiment performed by P. Farrell.



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#### 7.6. CHARACTERISTICS OF THE INHIBITOR

The above results are consistent with a number of hypotheses for the chemical nature of the inhibitor. It could be a new compound generated de novo by the interferon-treated cell extract. Alternatively it could be an oxidising agent, a hemin chelator, or dsRNA modified in some way to escape the high dsRNA control.

Inhibition occurred in the presence of either 25  $\mu$ M or 50  $\mu$ M hemin (results not shown). It is thus unlikely to involve complexing or degradation of hemin. Inhibition was not reversed by DTT, glucose, or fructose-1,6-diphosphate; compounds which reverse the inhibition due to oxidising agents (P. Farrell, personal communication 1976).

The resistance of the inhibitor to two degradatory enzymes was investigated. The choice of enzymes was limited to those whose activity could be prevented by an inhibitor, such that neither the enzyme nor its inhibitor would effect protein synthesis.

Trypsin complexed with soybean trypsin inhibitor does not effect the lysate. When the interferon and dsRNA-dependant inhibitor was treated with trypsin, at about 50  $\mu$ g trypsin per 1 mg cellular protein, at 30°C for 30 min no reduction in inhibition was observed (Fig. 44a). If the inhibitor has a protein moiety, it is resistant to trypsin under these conditions.

Micrococcal nuclease is a calcium-dependant nuclease (Cuatrecasas et al 1967), and does not effect the lysate providing a chelating agent is present (Pelham and Jackson 1976). Incubation with this nuclease at 280  $\mu$ g/ml in 5mM  $\text{CaCl}_2$ , partly degrades dsRNA as shown by a reduction in its ability to inhibit the lysate (Fig. 44b). When an inhibitor preparation was treated under the same conditions, it was completely destroyed (Fig. 44b). Thus it seems that the inhibitor contains a micrococcal nuclease sensitive moiety.

FIG. 44. Sensitivity of the inhibitor to a protease and a nuclease.

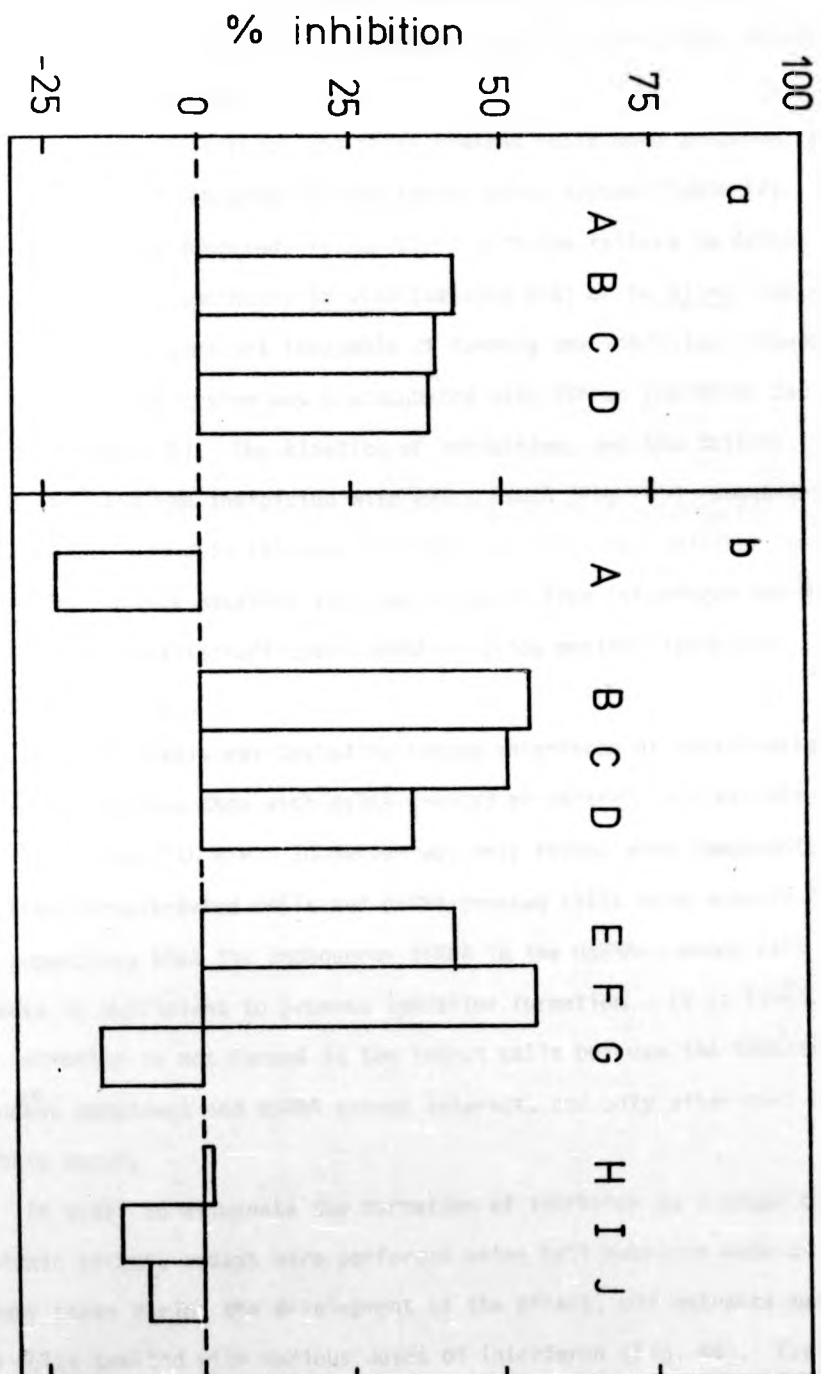
"Inhibitor" was formed as usual (section 2.3.9), and assayed after treatment with trypsin or micrococcal nuclease. The enzymes were inactivated before assay. Incorporation, after 30 min at 30°C, is expressed as a percentage inhibition, relative to the incorporation in the presence of a preincubation containing no dsRNA (0% inhibition).

(a) Sensitivity to trypsin. "Inhibitor" preparations formed by preincubating interferon-treated cell extract with 100 ng/ml dsRNA were digested with one-half volume of 200 µg/ml trypsin at 30°C for 30 min, before adding an equal weight of trypsin inhibitor and assaying in the lysate. "0% inhibition" was 13900 cpm.

	addition at 0 min.			addition at 30 min	
	"inhibitor"	trypsin	trypsin inhibitor	trypsin	trypsin inhibitor
A	-	-	-	+	+
B	+	-	-	+	+
C	+	+	-	-	+
D	+	+	+	-	-

(b) Sensitivity to micrococcal nuclease. dsRNA at 100 ng/ml in extraction buffer, an "inhibitor" preparation, or a "control" preparation (preincubation of control cell extract with 100 ng/ml dsRNA), were digested with one-half volume of 400 µg/ml nuclease and 15 mM CaCl<sub>2</sub> at 30°C for 30 min. K<sub>4</sub>EGTA was added to 12.5 mM before assaying in the lysate. The inhibition due to dsRNA was assayed in lysate without the addition of extra dsRNA.

	dsRNA	addition at 0 min			addition at 30 min	
		"inhibitor"	"control"	nuclease + CaCl <sub>2</sub>	EGTA	nuclease + CaCl <sub>2</sub>
A	-	-	-	-	+	+
B	+	-	-	-	-	-
C	+	-	-	-	+	+
D	+	-	-	+	+	-
E	-	+	-	-	-	-
F	-	+	-	-	+	+
G	-	+	-	+	+	-
H	-	-	+	-	-	-
I	-	-	+	-	+	+
J	-	-	+	+	+	-



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### 7.7. IS AN INHIBITOR FORMED IN VIVO?

If an inhibitor can be formed when dsRNA is added to an interferon-treated cell extract, then it is possibly formed in vivo, when dsRNA is added to interferon-treated cells.

Extracts of interferon and dsRNA treated cells were prepared, and tested without preincubation in the lysate assay system (Table 12). No inhibitor could be detected, in agreement with the failure to detect changes in protein synthesis in vivo (section 5.6) or in vitro (Table 10).

These cells were not incapable of forming the inhibitor, however. When the cell-free system was preincubated with ATP an inhibitor was generated (Table 12). The kinetics of inhibition, and the failure to greatly increase the inhibition with extra dsRNA (Fig. 45), suggest that the inhibitor formed in this way is identical with the inhibitor described before. It is thus possible that the extracts from interferon and dsRNA treated cells contain sufficient dsRNA to allow maximal inhibitor formation in vitro.

This hypothesis was tested by taking interferon or mock-treated cell extracts and mixing them with dsRNA-treated or control cell extracts, and incubating them with ATP. Inhibitor was only formed when components from both interferon-treated cells and dsRNA-treated cells were present (Table 13), suggesting that the endogenous dsRNA in the dsRNA-treated cell extracts is sufficient to promote inhibitor formation. It is likely that inhibitor is not formed in the intact cells because the interferon-dependent component and dsRNA cannot interact, and only after cell lysis can this occur.

In order to eliminate the formation of inhibitor as a cause of the cytotoxic effect, assays were performed using cell extracts made at various times during the development of the effect, and extracts made from cells treated with various doses of interferon (Fig. 46). Even as much as 3000 u/ml of interferon was inadequate to allow inhibitor formation



TABLE 12: Assay for inhibitor in extracts of interferon and dsRNA treated cells.

Treatment of cells		Incorporation (cpm) in reticulocyte lysate assay when cell extract added		
interferon (u/ml)	poly rI.rc (μg/ml)		without preincubation	with preincubation
			-dsRNA	+dsRNA
100	50	27130	5149	5488
100	0	25138	ND <sup>b</sup>	ND
0 <sup>a</sup>	50	25889	12314	10636
0	0	23768	ND	ND

Extracts were prepared from cells which had been treated with interferon for 18 hr and with poly rI.rc for 30-35 min. They were assayed without preincubation in a lysate containing 10 μg/ml dsRNA and 106 μCi/ml <sup>35</sup>S methionine. 3 μl aliquots were taken for acid precipitation after 30 min. They were then preincubated with an energy supply and 0 or 100 ng/ml dsRNA for 30 min at 30°C, before assaying in the presence of 10 μg/ml dsRNA and 53 μCi/ml <sup>35</sup>S methionine 3 μl aliquots were taken for acid precipitation after 30 min.

a) "mock-interferon" added.

b) ND = not done.

Details of the extracts are given in Expt. 1 of Table 10: they were assayed at 10-fold dilution in the lysate.

FIG. 45. Kinetics of inhibition of the lysate by preincubated extracts from dsRNA-treated cells.

Preincubations contained extracts from interferon or "mock-interferon" and poly rI.rC treated cells (see legend to Table 12), an energy supply, and 0 or 100 ng/ml dsRNA. After 30 min at 30°C the preincubations were diluted 10-fold into reticulocyte lysate containing 10 µg/ml dsRNA and 53 µCi/ml <sup>35</sup>S methionine. 3 µl aliquots were acid precipitated as required.

- Δ extraction buffer
- extract from control, dsRNA-treated cells, preincubated alone
- extract from control, dsRNA-treated cells preincubated with added dsRNA
- extract from interferon and dsRNA-treated cells, preincubated alone
- extract from interferon and dsRNA-treated cells, preincubated with added dsRNA.



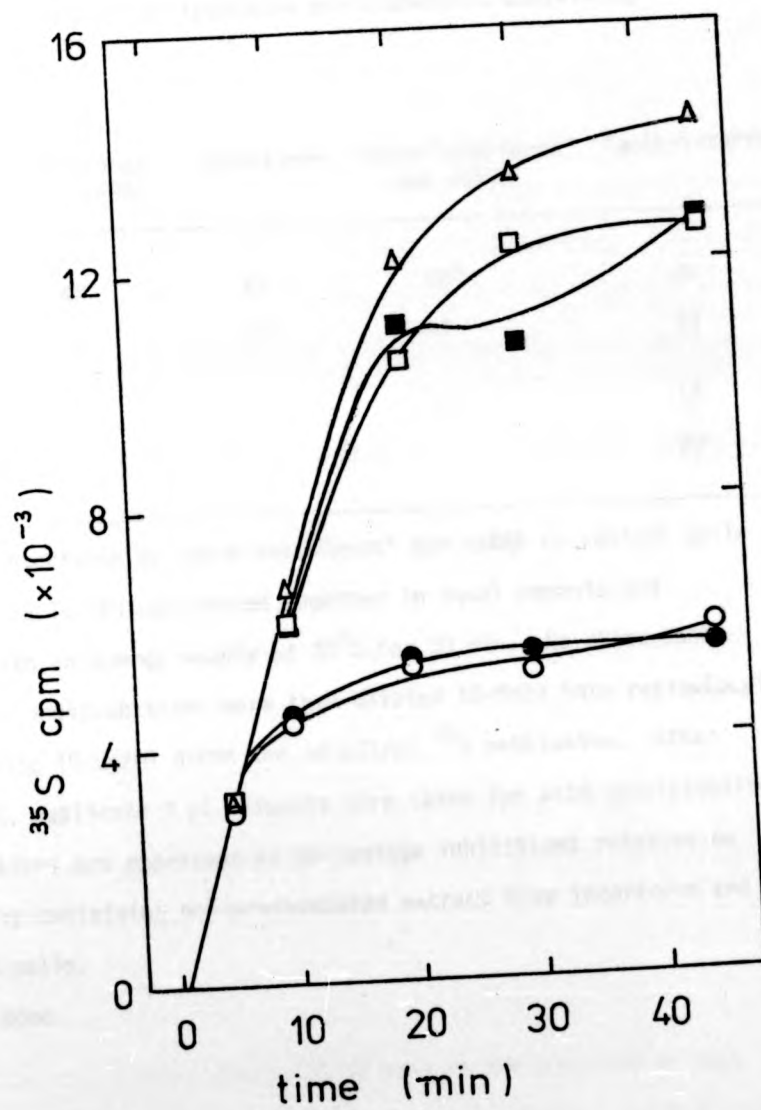


TABLE 13: Requirement for components from both interferon-treated and dsRNA-treated cells to form inhibitor in vitro

% inhibition of lysate by preincubations containing

extract B	extract A			
	interferon and dsRNA	interferon	"mock-interferon" and dsRNA	"mock-interferon"
interferon and dsRNA	46	47	ND <sup>a</sup>	36
interferon		10	41	18
"mock-interferon" and dsRNA			13	18
"mock-interferon"				20

Extracts from interferon or "mock-interferon" and dsRNA or control cells (see legend to Table 12) were mixed together in equal amounts and preincubated with an energy supply at 30°C for 30 min. No extra dsRNA was added. The preincubations were then diluted 10-fold into reticulocyte lysate containing 10 µg/ml dsRNA and 40 µCi/ml <sup>35</sup>S methionine. After 30 min at 30°C, duplicate 3 µl aliquots were taken for acid precipitation. The incorporations are expressed as percentage inhibitions relative to a control assay containing non-preincubated extract from interferon and dsRNA-treated cells.

a) ND = not done.

in dsRNA-treated cells, despite the fact that these cultures proceeded to loose 41% of cellular protein due to the cytotoxic effect. Inhibitor formation was readily detected when the extracts were preincubated (Fig. 46a) and could have been detected even if present in only 41% of the cells.

When extracts were made at various times after dsRNA treatment, no inhibitor could be detected (Fig. 46b). This excludes inhibitor formation at any time in the population of cells resistant to the toxic effect (section 5.3), but cannot exclude inhibitor formation shortly (less than 15 min) before cell loss from the monolayer. These extracts were also able to form inhibitor when incubated in vitro (data not shown).

If inhibitor formation was necessary for the toxic effect, changes in protein kinase activity might be seen. However, when interferon and dsRNA-treated cell extracts were labelled with ATPY  $^{32}\text{P}$ , under conditions known to phosphorylate proteins (section 6.7), and analysed on polyacrylamide gels, no changes were found (Fig. 47). However, no changes were detected when interferon-treated cell extracts were incubated in vitro with dsRNA and ATPY  $^{32}\text{P}$  (results not shown), suggesting that the protein kinase may not be able to modify L929 cell proteins under these conditions, perhaps because of prior phosphorylation by ATPY  $^{31}\text{P}$ . The changes which were seen (Fig. 47) were dependant on interferon but not dsRNA. Molecular weight markers were not run on this gel, but similar gels suggested that band X is in the range of 60-70,000 daltons, and band Y in the range 30-40,000 daltons.

The above experiments do not exclude the possibility that the inhibitor is present in vivo but is rapidly degraded during cell breakage. This possibility could only be adequately controlled for if conditions were found where the inhibitor could be detected in freshly-lysed cells.

**Fig.46.** No inhibitor in non-preincubated extracts of interferon and dsRNA treated cells.

Detergent extracts of cells treated with interferon and dsRNA were assayed, without preincubation, in reticulocyte lysate containing 10 µg/ml dsRNA and 59 µCi/ml <sup>35</sup>S methionine. After 30 min at 30°C, duplicate 3 µl aliquots were acid precipitated. The incorporation is expressed as a percentage inhibition, relative to a control containing interferon treated, but not dsRNA treated, cell extract (part a, 0% inhibition = 10800 cpm), or to a control containing extraction buffer (part b, 0% inhibition = 9500 cpm).

(a) Effect of concentration of interferon. Cultures were treated overnight with various concentrations of interferon or with "mock-interferon" (M), and then with 50 µg/ml poly rI.rC for 60-70 min, before making detergent extracts (see Expt. 2., Table 12). These extracts were assayed directly, or after preincubation with an energy supply for 30 min at 30°C. Preincubation had little effect on the control incorporation

● assayed without preincubation

△ assayed after preincubation

(b) Effect of time of dsRNA treatment. 24 cultures were treated with 300 u/ml interferon, and 4 with "mock-interferon", for 16-17 hr. After washing to remove serum, the cells were treated with 0 or 50 µg/ml poly rI.rC and, at various times, duplicate plates were taken and detergent extracts made. The extracts were assayed without preincubation and each point represents the percentage inhibition caused by a separate cell extract, plotted against the time of addition of poly rI.rC.

● cells treated with interferon and poly rI.rC

○ cells treated with interferon

■ cells treated with "mock-interferon" and poly rI.rC

□ cells treated with "mock-interferon"

All the extracts were assayed at 10-fold dilution in the lysate; details of those used for part(a) are given in Table 10 (Expt.2), and the toxicity in part(b) was normal.

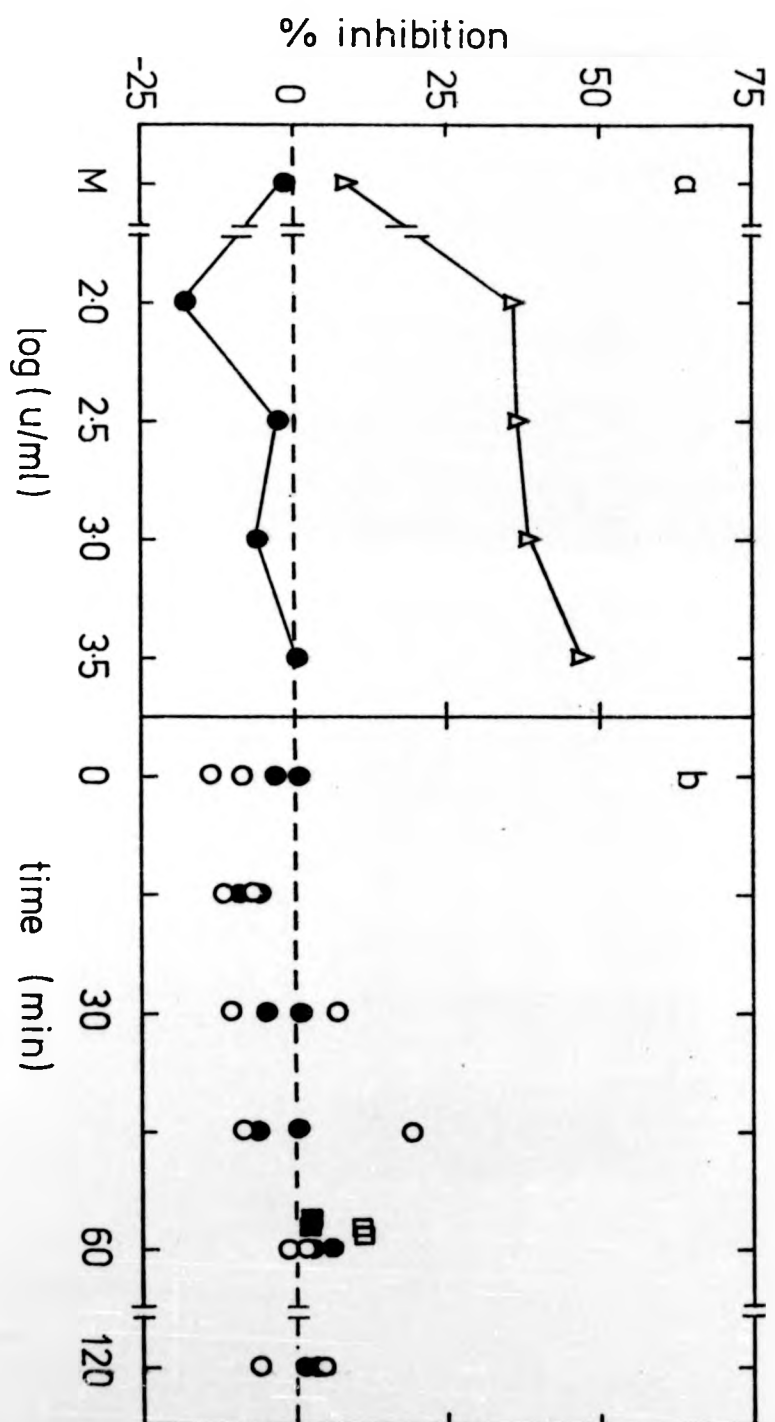


FIG. 47. Phosphorylation of proteins by extracts from interferon and dsRNA-treated cells.

Cells were treated overnight with 100 u/ml interferon or "mock-interferon", and then with 0 or 50 µg/ml poly rI.rC for 30-35 min (see Expt. 1 in Table 10). 90 µl of extract was incubated with 10 µl of water containing about 50 µCi of ATPY <sup>32</sup>P at about 8 Ci/m mole (i.e. about 65 µM), for 5 min at 37°C before removing two 5 µl aliquots for acid precipitation and treating the remainder with SDS. These samples were dialyzed exhaustively before analysing on a 7.5-30% gradient polyacrylamide gel (section 2.6.2). After electrophoresis at 16 mA for 14 hr the gel was dried without fixing, and exposed to Kodirex for 24 hr.

A, cells treated with interferon and dsRNA

B, cells treated with interferon

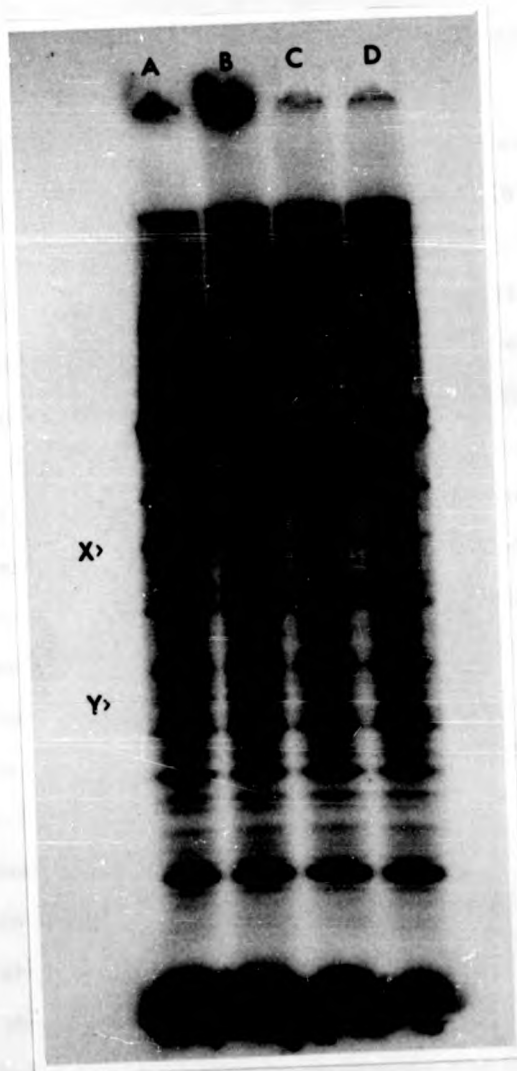
C, cells treated with "mock-interferon" and dsRNA

D, cells treated with "mock-interferon"

Each track was loaded with about 270000 hot TCA insoluble <sup>32</sup>P cpm. The bands marked "X" and "Y" were estimated to correspond to molecular weights of 60-70000 and 30-40000 daltons respectively, by comparison with molecular weight markers run on similar gels.

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### 7.8. DISCUSSION

A reticulocyte lysate assay has been developed which can detect preformed inhibitory factors but is not influenced by dsRNA. Using this assay, an inhibitor can be detected when interferon-treated cell extracts are incubated with dsRNA and ATP, but not when incubated with ATP alone. Control extracts cannot generate the inhibitor.

The lysate assay differs in several respects from the L cell extract assay used by Roberts *et al* (1976). When partly-purified inhibitor from an interferon-treated L cell S-100 (kindly provided by I.M. Kerr) was assayed in the lysate, it was not detectable at a 1000 fold dilution, although at this concentration it strongly inhibited L cell protein synthesis (I.M. Kerr, personal communication). This is possibly due to the presence in the lysate of higher concentrations of initiation factors, ribosomes, and so on, requiring more inhibitor for inactivation. On the other hand, it may reflect a different mechanism for inhibition.

Despite its lower sensitivity, use of the lysate facilitates an investigation of the mode of action of the inhibitor, because more is known about mechanism and control of protein synthesis in the lysate than in L cell extracts, and the system is highly active. The inhibitor acts at initiation (P. Farrell and J.A.C.), by preventing met tRNA<sub>F</sub> binding to the 40S subunit. This lesion is overcome by pure IF-E2, suggesting that the inhibitor inactivates that factor. Phosphorylation of the 35000 dalton subunit of IF-E2 has previously been correlated with inhibition by dsRNA, absence of hemin, and oxidising agents (P. Farrell, K. Balkow, I. Hunt and R. Jackson, personal communication 1975) and phosphorylated IF-E2 may be inactive in met tRNA<sub>F</sub> binding (B. Hardesty, personal communication 1976). In agreement with the proposed mechanism of inhibition by the interferon and dsRNA-dependant inhibitor, phosphorylation of this subunit was observed (Fig. 43). The absence of phosphorylation of the 67000 dalton protein during action of the L cell inhibitor suggests that this phosphorylation is not directly



involved in the dsRNA inhibition, but may represent a step in amplification of the response.

It is likely that this inhibitor is identical with the one recently described (Kerr et al 1976), which was formed in dialysed cell sap from interferon-treated cells by incubation with dsRNA and ATP. Indeed, such an inhibitor preparation (kindly provided by I.M. Kerr) was able to inhibit protein synthesis in the lysate assay, and the inhibitor described here inhibited protein synthesis in an L cell extract programmed with EMC mRNA (I.M. Kerr, personal communication 1976). The only differences in the preparations are in cell growth conditions (suspension or monolayer), source and dose of interferon, and method for making cell extracts. The conditions for forming the inhibitor, in terms of time, ATP and dsRNA requirements, are similar (Kerr et al 1976).

The identity of the inhibitor is uncertain. If its primary action is to phosphorylate IF-E2, one could postulate that the inhibitor is a protein kinase or an activator for a protein kinase present in the lysate. It is resistant to trypsin, and also to aminopeptidase and heat (I.M. Kerr, personal communication), suggesting it is an activator rather than a kinase. As well as being sensitive to micrococcal nuclease, it is of low molecular weight (Kerr et al 1976), and could thus be a modified nucleotide or oligonucleotide. In prokaryotic systems, guanosine tetraphosphate is an important coordinator of protein synthesis initiation and elongation and RNA synthesis (Travers 1976), and cAMP is a well known modulator of protein kinases in pro-and eukaryotes (Jost and Rickenberg 1971). In this case, however, cAMP can be ruled out, as the phosphorylation of IF-E2 is independent of cAMP (Traugh et al 1976).

The original hypothetical roles for the inhibitor, were that it mediated the antiviral effect and the interferon-enhanced toxicity of dsRNA (Kerr et al 1974b). The work presented here throws no light on the antiviral effect, but seems to exclude any involvement in the toxic effect. Cultures treated in such a way as to produce a marked toxic effect contained

undetectable levels of inhibitor; that is, the extracts were estimated to contain less than 5% of the amount of inhibitor that they were capable of forming in vitro (Fig. 46a and Fig. 41). The simplest explanation for this is that the dsRNA does not enter the cytoplasm in a form able to interact with the "inhibitor generating system". Presumably dsRNA formed intracellularly by a virus is able to cause inhibitor formation, giving rise to the effects observed by Friedman et al (1972a,b). However, the inhibitor formed in vivo in infected, interferon-treated cells must be shown to be identical with the one formed in vitro (when interferon-treated cell extracts are incubated with dsRNA), before the relevance of the inhibitor to the antiviral effect can be established.

The final point to arise from this work is the significance of the protein phosphorylation recently reported by other workers (Roberts et al 1976, Zilberstein et al 1976, Lebleu et al 1976). The consensus of opinion is that the phosphorylation of two proteins present in the ribosome wash fraction may be important in the control of translation (Roberts et al 1976, Zilberstein et al 1976) or nuclease action (Lebleu et al 1976). The phosphorylation of a protein of about 67000 daltons molecular weight is probably interferon-dependant, but only marginally dsRNA-dependant. On the other hand, the phosphorylation of another protein, of about 35000 daltons molecular weight, is dsRNA dependant, and also more or less dependant on interferon. Although Zilberstein et al (1976) have correlated these changes with a block in the formation of met tRNA<sub>F</sub>.40S initiation complexes, it was not clear whether these changes were causally related. The present work has shown that an interferon and dsRNA-dependant L cell inhibitor can prevent met tRNA<sub>F</sub>.40S formation in the reticulocyte lysate, at the same time causing phosphorylation of a 35000 dalton ribosome-associated protein. The same protein is phosphorylated under conditions of dsRNA-dependant inhibition of the

lysate, and has been shown to be identical with the small subunit of IF-E2 (P. Farrell, K. Balkew, T. Hunt and R. Jackson, personal communication 1975). This phosphorylation inactivates the factor (B. Hardesty, personal communication 1976). Thus it is probable that the 35000 dalton protein which is phosphorylated in L cells (Kerr et al 1976; Zilberstein et al 1976) or ascites tumour cells (Lebleu et al 1976), corresponds to this initiation factor, and is causally related to the inhibition of protein synthesis. Disagreement over the detailed pattern of phosphorylation probably results from variation in the level of prior phosphorylation with  $^{31}\text{P}$  orthophosphate and in the concentration of ATP used in the phosphorylation assay (Lebleu et al 1976).

SECTION 8  
GENERAL DISCUSSION

## 8. GENERAL DISCUSSION

Despite a survey of macromolecular synthesis and of ion transport rates, the molecular basis for the cytotoxic effect is unclear. It is apparent that protein synthesis is not inhibited, and no protein synthesis inhibitor can be detected in cell extracts. No other quantitative or qualitative changes were found, perhaps because a proportion of the cells resist the toxic effect. Other ~~works~~<sup>workers</sup> have found no correlation of toxicity with lysosomal activation (J. Werenne, G. Rousseau and J. Bartholeyns, personal communication 1976), and further investigation of the mechanism of cell death awaits a testable alternative hypothesis. The apparent absence of a protein synthesis inhibitor in dsRNA treated cells, when one is readily detected in dsRNA treated extracts, is circumstantial evidence that dsRNA does not enter the cell, although there are other explanations. If this is so, it is possible that dsRNA acts directly at the plasma membrane, causing cell lysis either by an ionic mechanism, or by activating an enzyme system capable of altering membrane lipids or proteins to produce an unstable membrane.

We have confirmed that interferon-treated cell extracts can form an inhibitor of protein synthesis, and characterised its action in the reticulocyte lysate. Clemens et al (1976) have recently reported a similar activity in a fraction derived from untreated ascites cells, and it is possible that such inhibitors are present in other cells and are modulated by dsRNA and hemin (C. Baglioni, personal communication 1976). These inhibitors prevent binding of initiator tRNA to the 40S ribosomal subunit, that is, a stage prior to mRNA binding. Metz (1975b) has suggested that interferon treatment might allow the cell to discriminate against high efficiency mRNAs, yet inhibition of initiation will actually promote the relative translation of high efficiency mRNA (Nuss et al 1975; Lodish 1974). Possibly the dsRNA-induced inhibition is sufficiently complete to slow viral replication to a stage where it

becomes uncoordinated or susceptible to other interferon dependant effects, such as impaired methylation or mRNA-specific nucleases. However, such speculation assumes that the in vitro inhibitor and the one operating in virus infected cells are the same, and this needs to be verified by co-purification and characterisation. If this is so, presumably interferon mRNA translation proceeds with very high efficiency, otherwise production of interferon by viruses in primed cells would not be possible.

The role of protein kinases in the inhibition has been clarified, and the interferon and dsRNA dependant inhibitor can be classed with dsRNA, hemin deprivation, and oxidising agents in terms of its effects on the reticulocyte lysate. Control of protein phosphorylation may be relevant in viral inhibition of host protein synthesis (Kaerlein and Horak, 1976), density dependant<sup>e</sup> inhibition of growth (Leader et al 1976), the control of enzyme systems (Jost and Rickenberg 1971), and control of transcription (Jost and Averbach 1975). It is thus possible that non-antiviral effects of interferon are modulated by protein kinases, especially since interferon is known to modulate cAMP levels (Weber and Stewart 1975). Interferon treatment may induce new enzyme systems, or modify existing ones, which are triggered by dsRNA to produce a protein kinase activator. The variety of interferon actions may be mediated by a variety of kinases. If the activator can be purified, it would be interesting to introduce it into intact cells and see which of the specific effects of interferon and dsRNA resulted.

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